The role of lysophospholipids in arrhythmogenesis

Bentham, Janet Margaret

Award date:
1986

Awarding institution:
University of Bath

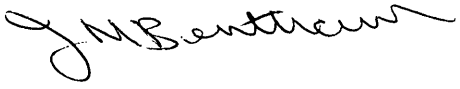
THE ROLE OF LYSOPHOSPHOLIPIDS IN ARRHYTHMOGENESIS

submitted by Janet Margaret Bentham

for the degree of PhD

of the University of Bath

1986.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For mum, dad and nana

ACKNOWLEDGEMENTS

I wish to thank both my supervisors, Dr. B. Woodward at the University of Bath, and Dr. A.J. Higgins at Pfizer Central Research, for all their help and guidance.

Many thanks are also due to the staff of the animal house for cooperation and assistance on numerous occasions.

I would like to thank two people at Pfizer Central Research, Anne Solca for all her help and patience with 'matters feline', and Mike Gwilt for his sense of humour!

Finally, grateful thanks to Mike Seed for the use of the word processor.

ABSTRACT

Lysophospholipid concentrations were shown to increase following coronary artery ligation of the isolated rat heart, prior to the development of arrhythmias, reaching a maximal concentration by 30 minutes post ligation. No changes in the parent phospholipids were seen.

Changes in the perfusate ionic composition that increased the severity of ischaemically induced arrhythmias caused an increase in control concentrations of lysophosphatidylcholine (LPC), a maximal LPC concentration being attained by either 20 minutes coronary artery ligation or increased perfusate calcium and decreased perfusate potassium concentrations. No significant changes were seen in tne parent phospholipids or lysophosphatidylethanolamine (LPE).

LPC was shown to be arrhythmogenic in the *in vivo* cat heart, final blood concentrations of 500μM LPC infused via the coronary artery producing severe arrhythmias and death. Palmitoylcarnitine (PAL), of similar structure to LPC, was also arrhythmogenic but apparently by a different mechanism as PAL appeared to produce ischaemia. This was unlikely to be due to a direct effect on platelet aggregation. The time courses of LPC and PAL induced arrhythmias were similar to those induced by coronary artery ligation.

Phospholipase A_2 activity was depressed in the ischaemic area of the isolated rat heart in both the homogenate and the mitochondria following 20 minutes coronary artery ligation, but not in the sarcolemma. This is possibly due to washout of inhibitory substances from the latter during the isolation procedure. LPC and PAL, when added exogenously, both showed an inhibitory effect on PLA_2 activity and thus may contribute to PLA_2 inhibition during ischaemia.

Reperfusion of the ischaemic isolated rat heart produced PLA_2 activation in the non-ischaemic, and to a lesser extent in the ischaemic, area. This may be due to free radical production as reduced glutathione limited these effects. It is also possible that endothelial damage produced by high flow rates may cause this change in PLA_2 activity as increased flow rates produced changes in PLA_2 activity that could be antagonised by vasodilators.

CONTENTS

PAGE

METHODS

PAGE

RESULTS

ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CAL	Coronary artery ligation
cAMP	Cyclic 3'5' adenosine monophosphate
CoA	Coenzyme A
EDTA	Ethylenediamine-tetra-acetic acid
GPC	Glycerophosphorylcholine
HPLC	High pressure liquid chromatography
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PAL	Palmitoylcarnitine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGI_2	Prostacyclin
PI	Phosphatidylinositol
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PS	Phosphatidylserine
PVC	Premature ventricular contractions
SEM	Standard error of the mean
TLC	Thin layer chromatography
TxA_2	Thromboxane A_2
VF	Ventricular fibrillation
VT	Ventricular tachycardia

INTRODUCTION

1.1 Coronary artery disease-clinical implications

Coronary artery disease is one of the most common single causes of death in the Western world with a third of all deaths in the USA in 1976 being attributable to it (Stallones 1980). Complications of coronary artery disease include angina pectoris, heart failure, myocardial infarction and sudden death. Although it had been suspected that an association existed between coronary artery obstruction, anginal pain and sudden death, it was not until 1912 that there was wide acceptance that myocardial infarction was produced by coronary thrombosis (Herrick 1912). In man atherosclerosis is the most frequent cause of coronary artery obstruction, atherosclerotic plaques being almost always present in cases of thrombosis (Schwartz, Chandler, Gerrity and Naito 1978). Flow changes resulting from plaque formation increase the risk of both endothelial damage and platelet contact with the vessel wall leading to platelet aggregation and thrombus formation (Azuma and Fukushima 1976). In patients suffering from acute myocardial infarction atherosclerosis is usually widespread and diffuse, however certain arterial segments appear predisposed to narrowing. The majority of thrombi are found in the left anterior descending, right and circumflex coronary

arteries in their first 2cm of length and at branch points (Davies, Woolf and Robertson 1976, Silver, Baroldi and Mariani 1980). Thrombi are rarely found in intramural coronary arteries (Roberts and Buja 1972, Ridolfi and Hutchins 1977).

In the majority of cases when a thrombus is found at post mortem it is in the vessel supplying the infarcted area (Silver et.al. 1980). However varying proportions of occlusive coronary thrombi have been found in patients with myocardial infarcts or at post mortem in sudden death cases, ranging from 36% (Branwood 1978) to 96% (Ridolfi and Hutchins 1977). It is thought that although thrombosis is a major cause of myocardial infarction other factors may be involved (MacAlpin 1974).

Sudden cardiac death, defined as where death occurs within an hour of being last seen alive is associated with a low incidence of thrombi (Reichenbach, Moss and Meyer 1977). Such death has been attributed to many different causes (review Podrid 1985), resulting in ventricular fibrillation (Armstrong, Duncan, Oliver, Julian, Donald, Fulton, Lutz, and Morrison 1972). Attention has also centred on the role of coronary artery spasm in producing sudden cardiac death (Oliva, Potts and Pluss 1973, Engel, Page and Campbell 1976). This is difficult to substantiate after the event although spasm has been seen during coronary arteriography with the use of contrast material (Oliva

et.al. 1973) and also at post mortem (El-Maraghi and Sealy 1980). The cause of this type of spasm is, as yet, unknown (review Kalsner 1982).

1.2 Metabolic events in myocardial ischaemia

Ischaemia is produced in myocardial tissue by a reduction in arterial flow due to a thrombus or spasm of the artery, or to an increase in myocardial work and oxygen demand greater than the arterial blood flow can supply (Jennings 1970). Ischaemia differs from hypoxia, where there is low oxygen delivery to the tissue with adequate perfusion, in that local accumulation of metabolites can occur due to reduced perfusion. In hypoxia there is no such accumulation as perfusion, and therefore washout, is maintained. The degree of reduction in arterial flow needed to induce ischaemia is not known because partial reductions may result in a heterogenous perfusion whose pattern cannot be determined (Steenbergen, DeLeeuw, Barlow, Chance and Williamson 1977). Following obstruction of a major coronary artery in the dog it has been shown that the blood flow in the subendocardium falls from 1-2 to 0-0.07ml/min/g, providing virtually no oxygen (Reimer and Jennings 1979).

When oxygen concentrations fall the rate of oxidative phosphorylation is reduced and an increase in the NADH/NAD ratio is produced. This leads to inhibition of aerobic metabolism; pyruvate dehydrogenase and

glyceraldehyde- 3- phosphate dehydrogenase both being inhibited by NADH (review Neely and Morgan 1974). The rate of the latter enzyme appears to be controlled primarily by product inhibition. Reduced ATP generation by oxidative phosphorylation gives an increased ADP/ATP ratio and thus an increase in glycolytic rate due to activation of phosphofructokinase, an important control point in glycolysis (Newsholme 1971/72). This increase in glycolysis is transient due to the accumulation of lactate (Liedtke, Hughes and Neely 1976). Lactate cannot be metabolised due to inhibition of pyruvate dehydrogenase, and it suppresses glycolysis, this being independent of changes in pH (Rovetto, Lamberton and Neely 1975). It also suppresses breakdown of glycogen to release glucose (Dobson and Mayer 1973). Thus even if substrate supply is plentiful it cannot be utilised.

Lactate accumulation leads to a reduction in intracellular pH due to the concomitant production of protons (review Neely and Morgan 1974, Hillis and Braunwald 1977) which also leads to glycolytic inhibition.

As very little ATP is being produced by oxidative phosphorylation and glycolysis the demand for ATP will exceed its production after a few minutes of ischaemia. To maintain the concentration of ATP the level of phosphocreatine falls (Braasch, Gudbjarnason, Puri, Ravens and Bing 1968, Garlick, Radda and Seeley 1979)

and this is followed by a continual decline in ATP (Braasch et.al. 1968, Allison, Ramey and Holsinger 1977, Jennings, Reimer, Hill and Mayer 1981). ADP content rises and the adenine nucleotide pool is rapidly destroyed with metabolism to inosine, hypoxanthine and xanthine (Jennings, Reimer and Steenbergen 1985).

Fatty acid oxidation, which normally provides the major energy source in the myocardium (Neely, Rovetto and Oram 1972), is reduced (Opie 1968) due to the increased NADH/NAD ratio which causes inhibition of one of the oxidative steps (Moore, Radloff, Hull and Sweeley 1980). There is also a decrease in the transport of fatty acids into the mitochondria (Wood, Sordahl, Lewis and Schwartz 1973) and therefore cytosolic accumulation of acyl CoA and acyl carnitine occurs (Idell-Wenger, Grotyohann and Neely 1978).

1.3 Electrophysiology of myocardial ischaemia

Following coronary artery occlusion there are three distinct phases of rhythm disturbances.

Phase 1 arrhythmias begin within minutes (Kaplinsky, Ogawa, Balke and Dreifus 1979) and terminate within 30 minutes. Phase 2 arrhythmias appear 12-24 hours later and phase 3 weeks after myocardial infarction. The mechanisms involved in phase 1 arrhythmias are thought to differ from phase 2 and 3 (Corr and Sobel 1979).

Phase 1 arrhythmias are thought to be due to re-

entry mechanisms operating (review Corr and Sobel 1980, Janse, Kleber, Capucci, Coronel and Wilms-Schopman 1986). Ischaemic cells have a reduced action potential duration, upstroke velocity and amplitude and a reduced resting membrane potential (Russell, Oliver and Wojtczak 1977), these being thought to lead to the production of arrhythmias. Re-entrant mechanisms rely on the presence of unidirectional conduction block and are promoted by slow conduction and inhomogeneities in refractory periods of adjacent areas, all of which occur in ischaemic tissue (Downar, Janse and Durrar 1977a, Elharrar, Foster, Jirak, Gaum and Zipes 1977, Russell et.al. 1977). Triggered activity, generated by early or delayed afterdepolarizations (Cranefield 1977) may also be a possible mechanism of ectopic impulse formation during ischaemia (Janse et.al. 1986).

The metabolic changes leading to these early arrhythmias are incompletely understood but it is thought that arrhythmogenic metabolites accumulate within the myocardium. The ischaemic heart, where metabolite retention occurs due to reduced flow, is more vulnerable to ventricular fibrillation than the hypoxic heart (Bagdonas, Stukey, Piera, Amer and Hoffman 1961).

Electrophysiological changes induced by ischaemia in an intact heart can be reversed by reperfusion with saline saturated with nitrogen. Under these circumstances

the tissue is still hypoxic suggesting that a deleterious metabolite is being washed out (Downar et.al. 1977). Venous blood from ischaemic regions is also capable of producing electrophysiological changes characteristic of ischaemic tissue in normoxic endocardium (Downar, Janse and Durrar 1977b).

There are many candidates in the search for the metabolites responsible for arrhythmogenesis including;

1) elevated extracellular potassium
2) catecholamines
3) elevated intracellular calcium
4) free fatty acids and their esters
5) prostaglandins
6) lysophospholipids

1.4 Possible mediators of early ischaemic arrhythmias

1) Elevated extracellular potassium

Extracellular potassium begins to rise within 15 seconds of the interruption of myocardial perfusion (Hill and Gettes 1980) reaching a plateau after 10-15 minutes (Hill and Gettes 1980, Hirche, Franz, Bös, Bissig, Lang and Schramm 1980). This increase in potassium is reversible upon reperfusion. During early ischaemia the extracellular potassium concentration may increase from 4mM to 10mM. This loss of potassium from the cells was

originally thought to be a result of reduced sodium / potassium ATPase activity, however evidence suggests that the sodium / potassium ATPase still operates (Kléber 1983) and therefore increased potassium efflux is likely to be the main cause (Rau, Shine and Langer 1977). There may be a role for ATP regulated potassium channels, which in cardiac muscle have been shown to produce an outward current which is inhibited by intracellular ATP (Noma 1983). During ischaemia, when intracellular ATP concentration falls these channels are likely to be activated and lead to potassium efflux. It has also been suggested that the increased potassium efflux and membrane conductance to potassium (Vleugels, Vereecke and Carmeliet 1980) is due to the decrease in fixed negative charge in the cell due to buffering of protons by intracellular proteins. This causes an outward movement of anions (lactate and inorganic phosphate) and cations (K^+)(Kléber 1984).

Increased extracellular potassium decreases the resting membrane potential and maximum velocity of the action potential upstroke (Morena, Janse, Fiolet, Krieger, Crijns and Durrer 1980). Hypoxia and acidosis accentuate these changes (Janse et.al. 1986).

Local differences in potassium concentration occur within the ischaemic tissue and these may be responsible for the inhomogeneity of refractory periods seen (Hill and Gettes 1980).

2) Catecholamine release

The autonomic nervous system is believed to influence arrhythmia production during ischaemia (review Verrier and Hagestad 1985).

Myocardial ischaemia is accompanied by increased blood catecholamine concentrations (Ceremużyński 1981) and in dogs this increase has been shown to precede the development of arrhythmias (Prakash, Parmley, Horvat and Swan 1972). In addition to release of noradrenaline and adrenaline from the adrenal medulla in ischaemia (Staszewska-Barczak and Ceremużyński 1968) local release of noradrenaline within heart tissue has been demonstrated (Carlsson, Abrahamsson and Almgren 1985).

It has been suggested that catecholamines are arrhythmogenic due to them elevating cellular cyclic AMP (cAMP) concentrations. This occurs due to binding of the catecholamine to beta receptors and subsequent adenylate cyclase activation. Evidence for the role of cAMP is conflicting. cAMP has been reported to be elevated in hearts succumbing to ventricular fibrillation in comparison to those with other types of arrhythmias (Corr, Witkowski and Sobel 1978) and dibutyryl cAMP decreases the ventricular fibrillation threshold independently of beta blockade (Lubbe, Podzuweit, Daries and Opie 1978). However other reports (Bricknell and Opie 1978, Kane, Morcillo-Sanchez, Parratt, Rodger and Shahid

1985) have not demonstrated a clear relationship between elevated cAMP concentrations and the production of arrhythmias. It is possible that cAMP may be the initiator of a chain of events that do not then require the continued presence of cAMP. cAMP is able to modulate the activity of the slow calcium channel in myocardial cells (Watanabe and Besch 1974) and increase calcium flux (review Green and Watanabe 1985) by phosphorylation of the calcium channel by cAMP dependent protein kinase (Osterreicher, Brum, Hescheler, Trautwein, Flockerzi and Hofmann 1982). This increased calcium influx may also be involved in the production of arrhythmias.

3) Elevated intracellular calcium

During ischaemia there is an abnormal rise in intracellular calcium (review Jennings et.al. 1985) leading to a reduction in the cell resting membrane potential. This can produce a flow of current to the normal tissue and initiate extrasystoles (Clusin, Buchbinder and Harrison 1983). Local release of catecholamines can stimulate calcium influx via the slow inward current and calcium efflux is depressed during ischaemia due to a decrease in active calcium transport. This is due to a reduction in cellular ATP and to reduced Na^{+} / Ca^{2+} exchange (Bersohn, Philipson and Fukushima 1982). Increased cellular calcium also regulates inter-cellular communication in cardiac muscle by controlling

the permeability of the intercalated discs (reviewed by DeMello 1982). Calcium increases the rate of healing of myocardial cells by a sealing process halting the spread of depolarising current (DeMello, Motta, Chapeau 1969) and intracellular injection of calcium decreases the electrical coupling between the cells (DeMello 1975). An increase in cellular calcium may therefore contribute to the impaired conduction seen in ischaemic tissue. Acidosis inhibits Na^+ / Ca^{2+} exchange (review Eisner, Allen and Orchard 1985) and thus ischaemic conditions may lead to elevated intracellular calcium producing electrophysiological abnormalities which may lead to arrhythmias.

Calcium accumulation has been suggested to be augmented by α-adrenergic activity (review Corr and Sharma 1984) with the number of α_1-receptors in ischaemic tissue increasing two fold (Corr, Shayman, Kramer and Kipnis 1981). Alpha blockade has proved to be antiarrhythmic (Davey 1980, Sheridan, Penkoske, Sobel and Corr 1980, Pogwizd, Sharma and Corr 1982) lending support to this hypothesis. Alpha adrenergic stimulation will also result in an increased slow inward current in ischaemic tissue (Miura, Inui and Imamura 1978). It is thought responses to catecholamines in ventricular tissue that are normally β receptor mediated are, in ischaemic tissue, α mediated (review Corr and Sharma 1984), such as the slow inward current (Miura et.al. 1978) and these

predispose to the development of arrhythmias.

4) Free fatty acids and their esters

Catecholamine release during ischaemia stimulates triglyceride lipolysis (Kruger, Leighty and Weissler 1967, Steinberg and Khoo 1977) from adipose tissue leading to elevated circulating free fatty acid concentrations (Oliver, Kurien and Greenwood 1968). These have been correlated with the incidence of arrhythmias (Kurien, Yates and Oliver 1971) however conflicting results have been found (Opie, Norris, Thomas, Holland, Owen and van Noorden 1971). It is possible that the absolute concentration of free fatty acid is less important than the ratio of free fatty acids to albumin (Willebrands, Ter Welle and Tasseron 1973) however ventricular fibrillation did not occur in isolated rat hearts if the ratio was increased to 12:1 (Evans, Opie and Shipp 1963).

Fatty acid metabolism by β oxidation is inhibited during ischaemia (review Neely and Morgan 1974) due to the increased NADH/NAD ratio and this leads to the accumulation of long chain fatty acyl derivatives such as acyl CoA and acyl carnitine (Liedtke, Nellis and Neely 1978). These are capable of exerting deleterious effects on myocardial membranes by detergent-like actions (Corr and Sobel 1981) and may therefore mediate part of the arrhythmogenic effects of free fatty acids.

5) Prostaglandins / Thromboxanes

Prostaglandins are synthesised from C_{20} unsaturated fatty acids released from membrane phospholipids by phospholipases. Although there is a large family of prostaglandins the physiologically important ones in ischaemia are thought to be prostacyclin (PGI_2) and thromboxane A_2 (TxA_2). Both PGI_2 and TxA_2 have been shown to be released from the ischaemic myocardium (Coker, Parratt, Ledingham and Zeitlin 1981).

TxA_2 is a potent vasoconstrictor and platelet aggregator, whilst PGI_2 is a potent vasodilator and inhibits platelet aggregation (Moncada and Vane 1978). TxA_2 and PGI_2 have been suggested to be pro- and anti-arrhythmic respectively (Coker et.al. 1981) and evidence for the suppression of ischaemically induced arrhythmias by increased prostacyclin production (Starnes, Primm, Woosley, Oates and Hammon 1982, Coker and Parratt 1984) or reduced thromboxane A_2 production (Smith, Lefer and Smith 1980, Coker 1984) has been obtained.

The action of thromboxane A_2 is possibly indirect by the aggravation of existing ischaemia by vasoconstriction and the formation of platelet aggregates. Due to the opposing actions of PGI_2 and TxA_2 it is likely that the balance between the two determines any pro- or anti-arrhythmic activity of the prostaglandins during myocardial ischaemia (Coker et.al. 1981).

Despite substantial evidence for the involvement of this balance in influencing the production of arrhythmias conflicting results have also been obtained. It has been reported that although infarcted canine myocardium has an increased synthetic capacity for TxA_2, its presence does not directly influence arrhythmogenesis or electrophysiological parameters late (3-7 days) during infarction (Kramer, Davies, Dean, McCluskey, Needleman and Corr 1985). TxA_2 has also been shown to have no involvement in the development of reperfusion arrhythmias in the dog (Burke, Antonaccio and Lefer 1982). These results may reflect differences in the aetiology of late and reperfusion arrhythmias respectively, in comparison to those produced by acute ischaemia.

6) Lysophospholipids

The accumulation of lysophospholipids in ischaemia due to the breakdown of membrane phospholipids is thought to have a role in mediating ischaemic damage and subsequent production of arrhythmias as detailed below.

1.5 LYSOPHOSPHOLIPIDS

1) Myocardial phospholipid composition

The major phospholipids of mammalian tissue are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which together comprise approximately 70% of the total phospholipid in human (Gloster and Harris 1969) and rat (Gloster and Harris 1970) myocardial fractions. Similar proportions have been found in rat sarcolemma (Tibbits, Sasaki, Ikeda, Shimada, Tsuruhara and Nagatomo 1981) and in pig myocardium (Shaikh and Downar 1981).

The remainder of the phospholipid is composed of phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin, cardiolipin (1-3-bis(phosphatidyl)-glycerol), and the lysophospholipids, lysophosphatidyl-choline (LPC) and lysophosphatidylethanolamine (LPE). PS and PI total less than 10% of total phospholipids but are thought to be important in calcium binding to sarcolemmal membranes (Philipson, Bers and Nishimoto 1980). PI is involved in receptor stimulated intracellular signalling (review Berridge 1984, Ransäs, Gjörstrup, Hjalmarson, Sjörren and Jacobsson 1986) however little of this work has been done in myocardial tissue.

Lysophospholipids are present at concentrations less than 5% of total phospholipids however they have strong cytolytic properties (Weltzien 1979) and small increases

in their concentration are thought to have profound effects on membrane structure and function.

2) Structure and properties of lysophospholipids

Lysophospholipids are produced by the hydrolysis of membrane phospholipids by phospholipases (FIG 1). Most membrane phospholipids are composed of a 3 carbon glycerol backbone to which two hydrophobic fatty acids and a polar, phosphate containing hydrophilic head group are esterified.

The action of phospholipase A_1 or A_2 produces a free fatty acid and a lysophospholipid (FIG 1). Lysophospholipids can also be produced by the hydrolysis of plasmalogens. Plasmalogens are glycerophospholipids with a 1-alkenyl group and can be deacylated by plasmalogenase or phospholipase A_2. The pathways for plasmalogen metabolism have not been completely elucidated but evidence does exist for different metabolism of plasmenylcholine and plasmenylethanolamine in guinea pig heart (Arthur, Page, Mock and Choy 1986).

Lysophospholipids are amphiphilic molecules, having a hydrophilic head group and a hydrophobic acyl group. This leads to their properties enabling interactions with biological membranes (Helenius and Simons 1975). At low concentrations they are incorporated into the lipid bilayer as monomers. This incorporation can change the physical properties of the bilayer (Fink and Gross 1984)

and the activity of membrane bound enzymes (Weltzien 1979). Incorporation of low concentrations of amphiphiles can expand the membrane area, possibly due to a transition from the gel to liquid state (Chapman, Urbina and Keough 1974) and this may cause conformational changes in ion channel proteins within the membrane.

Evidence exists that the activities of intrinsic membrane proteins are regulated by the phospholipid environment around the protein (Warren, Houslay, Metcalfe and Birdsall 1975) and thus the interactions of an amphiphile with this lipid annulus can potentially modify the protein function.

At higher concentrations amphiphiles aggregate into micelles in which the hydrophobic regions are clustered together in a core with the hydrophilic groups exposed externally to the aqueous environment. These micelles are able to incorporate into membranes and aggregate with the membrane lipids to form mixed micelles. This depletion of membrane phospholipids can produce a variety of functional changes and destroy the integrity of the membrane. In the heart this could lead to calcium influx and cell death (review Katz and Reuter 1979).

Compounds of amphiphilic nature such as lysophospholipids are therefore able to exert effects on membrane structure and function, and at sufficiently high concentrations can lead to disruption and loss of the ability of the cell membrane to act as a barrier.

3) Evidence for accumulation of lysophospholipids in ischaemia

Lysophospholipid accumulation has been shown to occur in the ischaemic pig myocardium (Shaikh and Downar 1981), increases of 60% in LPC and LPE being seen following 8 minutes coronary artery ligation. In the cat myocardium increases of 53% in LPC plus LPE have been reported in the ischaemic area following ten minutes coronary artery occlusion in comparison to controls (Corr, Snyder, Lee, Gross, Keim and Sobel 1982), LPC + LPE increasing from 4.9 +/- 0.27 to 7.5 +/- 0.33 nmol/mg protein, the ratio of LPC + LPE to the corresponding diacylphospholipids (PC + PE) increasing by 89% although there was no significant decrease in PC + PE. In an *in vitro* preparation of the dog heart, involving the production of global ischaemia by incubating tissue at 37°C in isotonic KCl under anaerobic conditions in sealed bags, a gradual increase of LPC with time from 1 to 24 hours has been demonstrated (Steenbergen and Jennings 1984).

Correlation of LPC + LPE concentration with the production of ventricular fibrillation following coronary artery ligation has been made in the anaesthetized cat, animals undergoing spontaneous ventricular fibrillation having the highest tissue LPC + LPE concentration (Corr, Sharma and Sobel 1983). One of the aims of this thesis

was to measure lysophospholipid concentrations in the isolated rat heart during ischaemia. This model is very well documented with a well defined progress of arrhythmias and therefore it should be possible to compare the two.

Increased lysophospholipid concentrations in the effluent from ischaemic cat myocardium *in vivo* have also been reported (Snyder, Crafford, Glashow, Rankin, Sobel and Corr 1981), the increase in LPC concentration being almost 100% following 10 minutes ischaemia. Difficulties arise in interpreting measurements of effluent concentration in this report due to the use of a washout technique to collect released LPC. It is unlikely that equilibrium would be established between the interstitial fluid and effluent during the washout however comparison with control washout for the percentage increase in LPC is valid.

4) Electrophysiological and arrhythmogenic properties of lysophospholipids

Lysophospholipids have been shown to produce marked electrophysiological changes when added exogenously to isolated tissue.

In solutions containing 0.4mM albumin at physiological pH, 1.2mM LPC induces changes resembling those seen in ischaemic tissue *in vivo* (Corr, Cain, Witkowski, Price and Sobel 1979). These changes are

reversible by superfusion with LPC free medium.

Clarkson and Ten Eick (1983) observed a decrease in resting membrane potential on exposure of cat papilliary muscle to 20-200μM LPC. This was associated with shortening of the action potential duration and a decrease in action potential amplitude. Again this could be reversed with LPC free perfusion. These changes were thought to be specific rather than being due to general membrane disruption as the membrane resistance increased and there was no change in the internal potassium concentration (Clarkson and TenEick 1983, Nakaya, Kimura and Kanno 1984).

In canine Purkinje fibres LPC produced concentration dependent decreases in the maximal diastolic potential, action potential amplitude and maximal rate of rise of phase 0 (Nakaya et.al. 1984). The highest LPC concentration used, 80μM, reduced action potential duration and increased the rate of spontaneous discharge. This was also seen by Arnsdorf and Sawicki (1981). Such abnormal automaticity is known to occur in partially depolarised Purkinje fibres on the border of the infarct zone (Dangman and Hoffman 1983).

The electrophysiological effects of LPC are enhanced by acidosis, Purkinje fibres having a three fold increase in sensitivity to LPC on reduction of pH from 7.4 to 6.7 (Corr, Snyder, Cain, Crafford, Gross and Sobel 1981) which was not due to increased cellular LPC incorporation

(Gross, Corr, Lee, Saffitz, Crafford and Sobel 1982). LPC is capable of producing changes in electrophysiological characteristics *in vitro* that have been shown to occur in ischaemic tissue *in vivo*, changes that its major metabolites glycerophosphorylcholine and free fatty acids do not produce (Corr et.al. 1979). Albumin binding of LPC appears important, with 10 fold greater LPC concentrations needed to produce the same effects in the presence of albumin (Corr et.al. 1981). It has been suggested that LPC acts as a non specific depressant of conductance as membrane resistance at potentials near the normal resting potential increased in cat papilliary muscle and LPC had an inhibitory effect on time dependent potassium conductance (Clarkson and Ten Eick 1983). 10μM LPC has been shown to produce an increased conduction time and spontaneous firing when perfused through the isolated rabbit heart (Nakaya, Ozaki, Kimura, Gotoh and Kanno 1984) and has been shown to produce arrhythmias in the isolated hamster (Man and Choy 1982) and rabbit (Bergmann, Ferguson and Sobel 1981) hearts.

Again the free concentration of LPC appears to be important as Man and Choy found a 10 fold increase in LPC concentration was required to achieve the same effects in the presence of albumin.

The critical micelle concentration, the concentration at which LPC forms micelles as opposed to

remaining as monomers, was thought to be related to the arrhythmogenic nature of the LPC (Bergmann et.al. 1981) but more recent studies have cast doubt on this because different acyl chain length LPC species with different critical micelle concentrations were shown to have similar arrhythmogenic properties (Man, Wong and Choy 1983). The type of polar head group and the degree of unsaturation of the acyl group also bore little relation to their arrhythmogenic action.

LPC has been shown to produce arrhythmias in isolated rat hearts, the ability to produce ventricular fibrillation being dependent on LPC concentration and perfusion time. This generation of fibrillation was dependent on the presence of external calcium however it did not appear to be dependent on the slow inward current as verapamil did not have a protective effect (Man and Lederman 1985).

Thus lysophospholipids have been shown to be arrhythmogenic in a number of isolated heart preparations. One of the aims in this thesis was to assess the arrhythmogenic effects of LPC in an *in vivo* model to see if arrhythmias could be produced in a model more physiologically relevant to the clinical situation.

5) Effects of lysophospholipids on ion transport systems

Lysophosphatidylcholine has been shown to have effects on ion transport and conductance in a number of systems.

Potassium efflux is increased in isolated rabbit hearts by 10μM LPC (Nakaya and Ozaki et.al. 1984). Although this increase was only slight due to continual perfusion it may represent a marked extracellular potassium accumulation if perfusion ceases as during ischaemia. The same workers also looked at the effect of LPC on intracellular potassium activity in canine Purkinje fibres but found no decrease during perfusion with 160μM LPC (Nakaya, Kimura and Kanno 1984), supporting the results of Clarkson and Ten Eick (1983). This may be due to the high intracellular potassium concentration, where small decreases sufficient to significantly increase extracellular potassium concentration would not be detectable. Lysophospholipids have also been shown to increase potassium efflux from erythrocytes (Lawrence, Moores and Steele 1974) and therefore any accumulation of lysophospholipids in ischaemia may contribute to the potassium efflux seen.

Inhibition of sarcolemmal Na^+ / K^+ ATPase by 10-60μM LPC has been shown (Karli, Karikas, Hatzipavlou, Levis and Moulopoulos 1979), this inhibition being reversed by

incubation with albumin. LPC is thought to act specifically at the sodium binding site which may contribute to the decrease in the Vmax of the upstroke seen on treatment of isolated tissue with LPC.

Calcium transport is also affected by LPC. In cultured rat cardiac myocytes LPC at 60-80μM is able to increase the rate of bidirectional calcium flux whilst at 100μM there is an increase in total cell calcium concentration (Sedlis, Corr, Sobel and Ahumada 1983). The increase in cell calcium seen after 5 minutes perfusion with 100μM LPC could be reversed by washing and was prevented by verapamil preincubation. Accumulation of calcium over longer periods of 100μM LPC exposure could not be reversed or prevented by verapamil and was accompanied by irreversible morphological changes. Calcium accumulation induced by LPC has also been shown in pigeon erythrocytes (Lee, Ting and Vidaver 1986). The calcium accumulation may occur through the slow calcium channel (Corr and Snyder et.al. 1982). LPC has been shown to increase the cAMP content of myocardium by stimulation of adenylate cyclase (Ahumada, Bergmann, Carlson, Corr and Sobel 1979) and this can cause elevation of the inward calcium current by cAMP dependent phosphorylation of the ion channel (Watanabe and Besch 1974).

Sodium / calcium exchange has been shown to be inhibited in sarcolemma isolated from ischaemic hearts and in sodium loaded canine sarcolemmal vesicles treated

with 30μM LPC (Bersohn and Philipson 1983). Although the Na^+ / Ca^{2+} exchange normally operates in the direction of calcium extrusion from the cell it is reversible (Bridge and Bassingthwaite 1982) thus if LPC causes inhibition of Na^+ / K^+ ATPase and produces an increased internal sodium concentration calcium influx could occur through Na^+ / Ca^{2+} exchange.

LPC has been shown to affect Ca^{2+}/ Mg^{2+}ATPase activity in human erythrocyte membranes, with long chain (C_{16} and C_{18}) unsaturated LPC molecules being stimulatory. These effects were highly dependent on the nature of the hydrophobic group and chain length (Tokumura, Mostafa, Nelson and Hanahan 1985).

LPC-induced changes may contribute to the changes seen in ion conductances during ischaemia. Recent evidence has pointed to a role of lysophospholipids in increasing α receptor number (Sharma, Ahumada, Sobel and Corr 1983), a change that also occurs in ischaemia (Corr, Shayman, Kramer and Kipnis 1981) and is thought to be pro-arrhythmic (Davey 1980).

6) Evidence for the site of action of lysophospholipids

Although the effects of exogenously added lysophospholipids on tissue excitability and function have been extensively studied this may not reflect their

effects when liberated endogenously. When membrane hydrolysis is carried out *in vitro* changes in fluidity do not occur unless the products are washed out of the membrane (Storch and Schachter 1985). The source of increased lysophospholipids during ischaemia is uncertain although there is evidence for it having a vascular or endothelial source. Increased LPC concentrations have been found in the coronary effluent from rat hearts (Stam and Hülsmann 1981) although this was under conditions of hormonal stimulation (noradrenaline or glucagon) rather than ischaemia. Venous effluent LPC concentrations increase during ischaemia (Snyder et.al. 1981) which could indicate vascular production or movement to the extracellular space (Stein and Stein 1965). Extracellular addition of LPC to isolated tissues would be expected to mimic closely the effects of LPC in ischaemia if it does act extracellularly. LPC in concentrations known to occur in ischaemia does exert similar electrophysiological changes to those seen in ischaemia. Exogenously added LPC has been shown to incorporate into isolated tissues, the concentrations in the sarcolemma being the greatest . Incorporation of 1.5nmol LPC / mg protein, which was less than 2% of the total phospholipids, caused marked electrophysiological changes (Gross et.al. 1982).

LPC is unlikely to be distributed homogenously within the cell, due to its lipid nature, therefore concentrations measured in ischaemic tissue are likely to

be an underestimate of the membrane concentration. As the sarcolemma of the rat heart comprises only 4% of its total membrane surface (Anversa, Olivetti, Melissari and Loud 1980) cellular concentrations probably give a very inaccurate measurement of the concentration in the sarcolemma.

The effects of LPC when added exogenously do not require incorporation of micelles (Bergmann et.al. 1981) although micelles may act as a reservoir providing monomers (Weltzien 1979). It is assumed that on initial exposure of a membrane to LPC monomers will incorporate into the outer layer of the lipid bilayer but then penetrate into the inner layer. For mammalian erythrocytes the rate constant for this movement is less than $0.03 hr^{-1}$ (Bergmann, Dressler, Haest and Deuticke 1984).

It is therefore possible that LPC produced in the vasculature during ischaemia could incorporate into cell membranes of myocytes and so alter their functional properties.

1.6 Production and metabolism of lysophospholipids

The major pathways of LPC metabolism are illustrated in FIG 2. Under normal physiological conditions phospholipids are undergoing continual turnover (review van den Bosch 1980) but accumulation of LPC does not occur because any produced is reacylated to PC or hydrolysed to glycerophosphorylcholine. As LPC accumulation has been shown to occur in ischaemia the relative rates of these processes must change. Increased production of LPC by phospholipase A_2 or decreased metabolism by lysophospholipases or the reacylating enzymes could all lead to increased LPC concentrations.

1) Phospholipase A_2 (EC 3.1.1.4)

Phospholipase A_2 (PLA_2) activity leads to the hydrolysis of a diacylphospholipid at the C-2 position to produce a lysophospholipid and a free fatty acid (FIG 2).

Two types of PLA_2 are commonly found, membrane bound and soluble forms.

Membrane bound enzymes are integral proteins in the plasma, mitochondrial and Golgi membranes, have a neutral to alkaline pH optimum and require calcium for activity (van den Bosch 1980). Membrane bound PLA_2 enzymes were thought to depend on calmodulin for their calcium dependent activity (Wong and Cheung 1979, Moskowitz, Shapiro, Schook and Puszkin 1983, Moskowitz, Andrés,

Silva, Shapiro, Schook and Puszkin 1985) however other reports have found no role for calmodulin (Withnall, Brown and Diocee 1984, Watanabe, Hashimoto, Teramoto, Kume, Naito and Oka 1986) and it is now thought likely that the effects of calmodulin and its antagonist trifluoperazine are due to non specific interactions with the membrane phospholipids.

Soluble phospholipases A_2 are thought to be lysosomal enzymes with no requirement for calcium and an acidic pH optimum (van den Bosch 1980) although there has been a recent report of a plasmenylcholine specific PLA_2 in the cytosolic fraction of the guinea pig heart with an alkaline pH optimum and a requirement for calcium (Arthur et.al.1986). A calcium independent cytosolic plasmalogen specific PLA_2 has also been identified (Wolf and Gross 1985).

Membrane bound PLA_2 enzymes are thought to be important in the production of lysophospholipids as they are active at physiological pH.

Activation of PLA_2 in the isolated cat heart during ischaemia has been reported (Shaikh and Downar 1985) however as small changes of specific radioactivity of labelled phospholipids were quoted with no indication of standard errors it is difficult to draw firm conclusions. In subcellular fractions from ischaemic portions of the isolated rabbit heart no change in assayable PLA_2 activity was seen (Corr and Sobel 1981). Hseuh, Isakson

and Needleman (1977) showed an increase in prostaglandin and fatty acid release during global ischaemia however their protocol does not rule out the role of reperfusion in this release. Activation of brain PLA_2 in ischaemia has been shown but this was only a transient activation persisting for a maximum of one minute ischaemia after which time the activity was depressed (Edgar, Strosznajder and Horrocks 1982). Work postulating activated PLA_2 during ischaemia has used indirect measurements by assaying prostaglandin production (Hsueh et.al. 1977) as this was thought to be the rate limiting factor in prostaglandin production (Flower 1978). However as arachidonic acid, the precursor of the prostaglandins, can also be released by the action of phospholipase C and diglyceride lipase (Bell, Kennerly, Stanford and Majerus 1979) and the rate of prostaglandin synthesis in one system was shown to be dependent on the rate of reacylation of the liberated fatty acid (Korner, Hausmann, Gemsa and Resch 1984) care must be taken in interpreting indirect measurements of phospholipase activity.

The activities of lysophospholipase and the reacylating enzyme lysophosphatide acyltransferase are both considerably greater than PLA_2 activity (Gross and Sobel 1981a, Gross and Sobel 1982, Korner et.al. 1984) and therefore it has been suggested that changes in these activities may be more important in affecting

lysophospholipid concentrations than changes in PLA_2 activity.

One aim in this work was to measure phospholipase A_2 activity in the isolated rat heart and therefore attempt to see if its activity does increase during ischaemia.

2) Lysophospholipase (EC 3.1.1.5)

Lysophospholipase hydrolyses the remaining fatty acyl ester to release glycerophosphorylcholine and a free fatty acid from LPC (FIG 2).

Lysophospholipases are not calcium dependent, have a pH optimum of 6-8 and do not hydrolyse diacylphospholipids (Gross and Sobel 1983). The effects of ischaemic conditions on their activity have been studied. Microsomal lysophospholipase is inhibited 81% by a reduction in pH to 6.5 from 7.4 (Gross and Sobel 1982), and cytosolic lysophospholipase is inhibited competitively by palmitoylcarnitine with a Ki of 10-11μM (Gross and Sobel 1983). Conditions occuring in the ischaemic myocardium are therefore likely to contribute to a decrease in lysophospholipase activity.

3) Lysophosphatidylcholine acyltransferase (EC. 2.3.1.23)

Lysophosphatidylcholine acyltransferase reacylates

LPC, with acyl CoA as the acyl group donor, producing a diacylphospholipid.

Inhibition of rabbit ventricular microsomal acyltransferase by glycerophosphorylcholine (GPC) has been shown (Gross and Sobel 1981b). In brain microsomes acyl CoA will also inhibit acyltransferase, this inhibition being overcome by high LPC concentrations (Deka, Sun and MacQuarrie 1986) therefore the ratio of acyl CoA to LPC in ischaemia could modulate the activity of this enzyme. In the hamster heart all reacylation of LPC is thought to be carried out by the acyltransferase, with no transacylase being detected (Savard and Choy 1982).

Acyltransferase was not detected in rabbit myocardial cytosol (Gross and Sobel 1981a) however as palmitoyl CoA was used as the acyl group donor and an acyltransferase specific for polyunsaturated fatty acids has since been identified in rabbit cardiac cytosol (Needleman, Wyche, Sprecher, Elliott and Evers 1985) this may explain the apparent lack of acyltransferase activity.

4) Lysophosphatidylcholine transacylase

Lysophosphatidylcholine transacylase produces PC and GPC from two molecules of LPC.

Rabbit myocardial cytosol has been found to possess this activity (Gross and Sobel 1981a) and the same enzyme protein may also carry out lysophospholipase activity. An

acyl enzyme intermediate is formed which subsequently undergoes nucleophilic attack by either H_2O or LPC to produce lysophospholipase or transacylase activity respectively. Low concentrations of palmitoylcarnitine (20μM) have been shown to inhibit this transacylase (Gross and Sobel 1981a, Gross, Drisdel and Sobel 1983). Inhibition by reduced pH was also seen, therefore the conditions in ischaemia are likely to cause a reduction in LPC transacylase activity.

1.7 Membrane phospholipid changes during myocardial ischaemia

Although it is assumed that lysophospholipid production is due to membrane phospholipid hydrolysis the changes in LPC concentration do not always correlate with changes in the membrane phospholipids (Steenbergen and Jennings 1984), no significant changes in diacylphospholipids being seen after short periods of ischaemia when significant increases in lysophospholipids are evident (Shaikh and Downar 1981, Corr et.al. 1982). However as lysophospholipids comprise less than 5% of the total phospholipids the decrease in diacylphospholipids occuring when lysophospholipids increase significantly would not be readily detectable.

In a number of models phospholipid concentration has been shown to decrease after longer periods of time.

After 30 minutes of ischaemia a 16% decrease was seen in the phospholipid content of dog sarcoplasmic reticulum (Yanagishita, Katagiri, Kitsu, Geshi, Konno, Tanno, Akiyama, Sekita, Kobayashi and Niitani 1985) and in other experiments in dog myocardium a similar decrease was also seen after 3 hours of ischaemia (Chien, Han, Sen, Buja and Willerson 1984). This phospholipid decrease is predominantly in the PC and PE species (Man, Slater, Pelletier and Choy 1983) and may represent the involvement of lysosomal enzymes due to the loss of lysosomal integrity (Hoffstein, Weissman and Fox 1976, Wildenthal 1978).

1.8 Phospholipid metabolism during reperfusion of the ischaemic myocardium

Reperfusion of the myocardium after prolonged ischaemia (>20 minutes) leads to extensive myocardial necrosis (Jennings et.al. 1985) accompanied by severe arrhythmias of rapid onset (reviewed by Manning and Hearse 1984). There is evidence of sarcolemmal (Jennings and Ganote 1974) and cell (Hearse, Humphrey and Chain 1973) disruption. This cell disruption and irreversible damage has been shown to correlate with the phospholipid depletion in the sarcolemma and reflects the loss of the ability of the membrane to act as a permeability barrier, leading to massive calcium influx and cell death (Chien,

Abrams, Serroni, Martin and Farber 1978).

Reperfusion of the myocardium may potentiate the phospholipid breakdown that occurs in ischaemia by washing out the products of this breakdown (Swoboda, Fritzsche and Hasselbach 1979, Katz 1982).

During reperfusion oxygen derived free radicals are produced (Gauduel and Duvelleroy 1984, McCord 1985). These highly reactive species have a very short half life. Under physiological conditions the natural cellular free radical scavengers such as catalase and superoxide dismutase are able to reduce the free radicals and prevent damage to the cell. Under ischaemic conditions it is thought that there is a decrease in intracellular scavengers and these are not able to metabolise the radicals formed on subsequent reperfusion.

A major component of cell injury due to free radicals is lipid peroxidation. Unsaturated fatty acids in membrane phospholipids react with the free radicals to produce an alkyl radical and from this a lipid peroxy free radical. This produces a disorganisation of membrane structure and a disturbance of normal function (Noronha-Dutra and Steen 1982). Lipid peroxidative injury can be potentiated by amphiphiles (Mak, Kramer and Weglicki 1986) therefore the production of lysophospholipids during ischaemia would be expected to enhance reperfusion injury due to peroxidation of the sarcolemma.

Enhanced lysophospholipid production by lysosomes

during free radical generation occurs *in vitro* (Weglicki, Dickens and Mak 1984). Significant increases in LPC plus LPE were accompanied by decreases in PC and PE and a reduction in lysosomal integrity. It is thought that peroxidised phospholipids are better substrates for phospholipases (Sevanian, Stein and Mead 1981) and thus initial free radical attack on the membranes, which may be potentiated by LPC, can lead to increased susceptibility to phospholipase and further increases in LPC concentration.

Any increase in LPC during ischaemia may therefore cause an increase in severity of damage during reperfusion.

1.9 AIMS

1) Measurement of lysophospholipid concentrations during ischaemia

One objective was to develop a method of phospholipid extraction, separation and quantification to use with small quantities of tissue obtained from the isolated rat heart. The isolated rat heart was used due to simplicity of the model and because the time course and duration of the arrhythmias produced by coronary artery ligation are well defined. The arrhythmias could then be correlated with any changes in lysophospholipid concentration.

The changes in the two lysophospholipids LPC and LPE, and their parent phospholipids, PC and PE, were measured during ischaemia produced by coronary artery ligation.

Changes in the perfusate ionic composition that alter the time course of coronary artery ligation induced arrhythmias were also examined for their effects on lysophospholipid concentrations.

2) Effects of lysophospholipids in vivo

Although work has been done on the effects of lysophospholipids on isolated tissues little work has been

done on their effects *in vivo*.

An anaesthetized cat model was used to determine the effect of lysophospholipids when infused directly into the coronary circulation via the left anterior descending coronary artery, and when infused into the left ventricular wall. This may show differences between the effect of LPC in the vasculature and interstitially. The effect of palmitoylcarnitine (PAL) was similarly determined. PAL is a long chain acyl carnitine which has been shown to accumulate during ischaemia and has a similar structure and therefore a similar amphiphilic nature to LPC (FIG 3). Effects displayed by LPC due to these amphiphilic properties are likely to be also displayed by PAL.

A major metabolite of LPC, glycerophosphorylcholine (GPC), does not show this amphiphilic structure and properties (FIG 3) and the effects of GPC were therefore studied as a control.

The effects of LPC and PAL were compared with the effects of coronary artery ligation. This may provide information on whether accumulation of either LPC or PAL could be involved with the development of arrhythmias *in vivo*.

3) Measurement of phospholipase A_2 activity

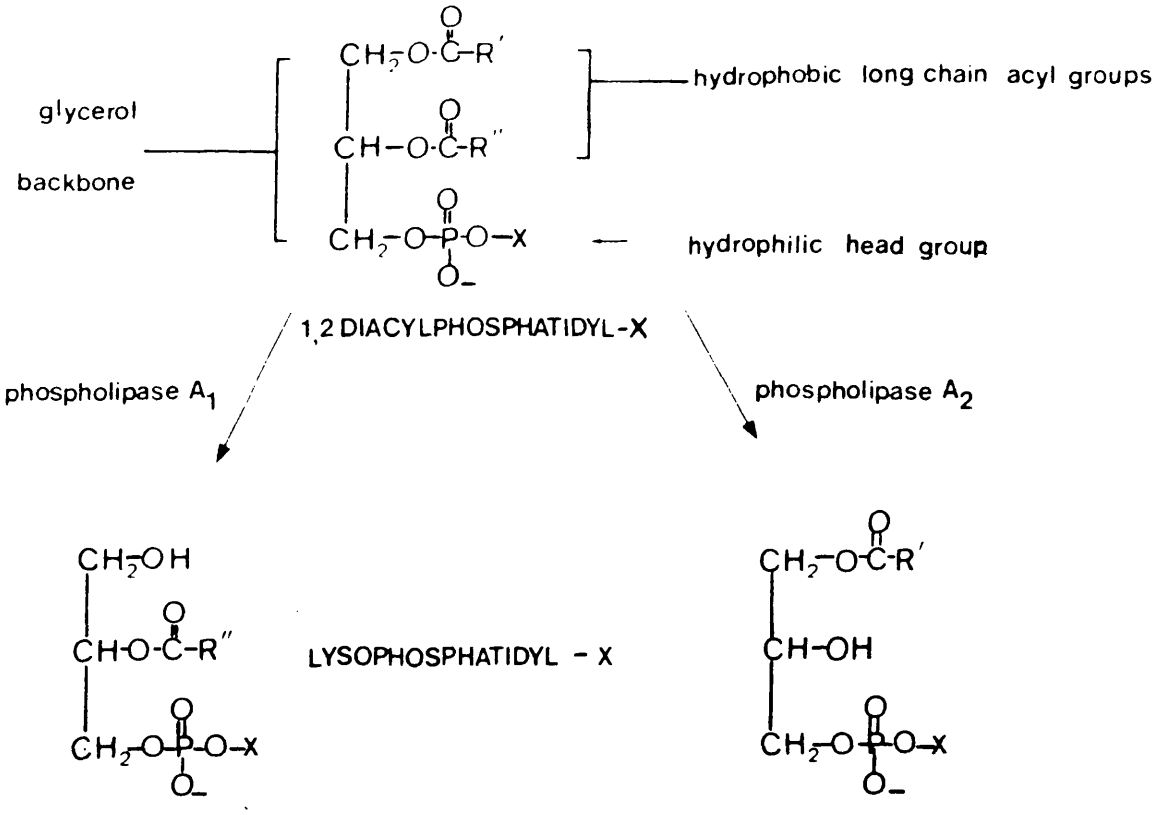
Increased phospholipase A_2 activity may contribute

to the increased LPC concentrations that have been reported during ischaemia. Therefore an assay was developed and characterised to allow measurement of phospholipase A_2 activity in homogenates and subcellular fractions of the isolated rat heart.

This allowed measurement of phospholipase A_2 activity during ischaemia at times when LPC concentration had been shown to rise, correlations of phospholipase A_2 activity with the concentration of LPC and the presence of arrhythmias could thus be made.

The activity of phospholipase A_2 in tissue samples from the anaesthetized cat was also measured to determine the effect of coronary artery ligation and infusion of LPC and PAL on the activity. This was compared with the effect of LPC and PAL on phospholipase A_2 *in vitro*.

Reperfusion of the ischaemic myocardium has been reported to be accompanied by phospholipid degradation (Jennings and Ganote 1974) and lysophospholipid production (section 1.8). The effect of reperfusion of the ischaemic isolated rat heart on phospholipase A_2 activity was determined to determine any possible role of phospholipase A_2 in this phospholipid degradation.

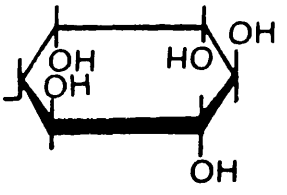


—X HEAD GROUPS FOUND IN MAJOR PHOSPHOLIPIDS:

$-CH_2CH_2\overset{+}{N}(CH_3)_3$ CHOLINE

$-CH_2CH_2NH_2$ ETHANOLAMINE

$-CH_2CH(NH_2)COOH$ SERINE

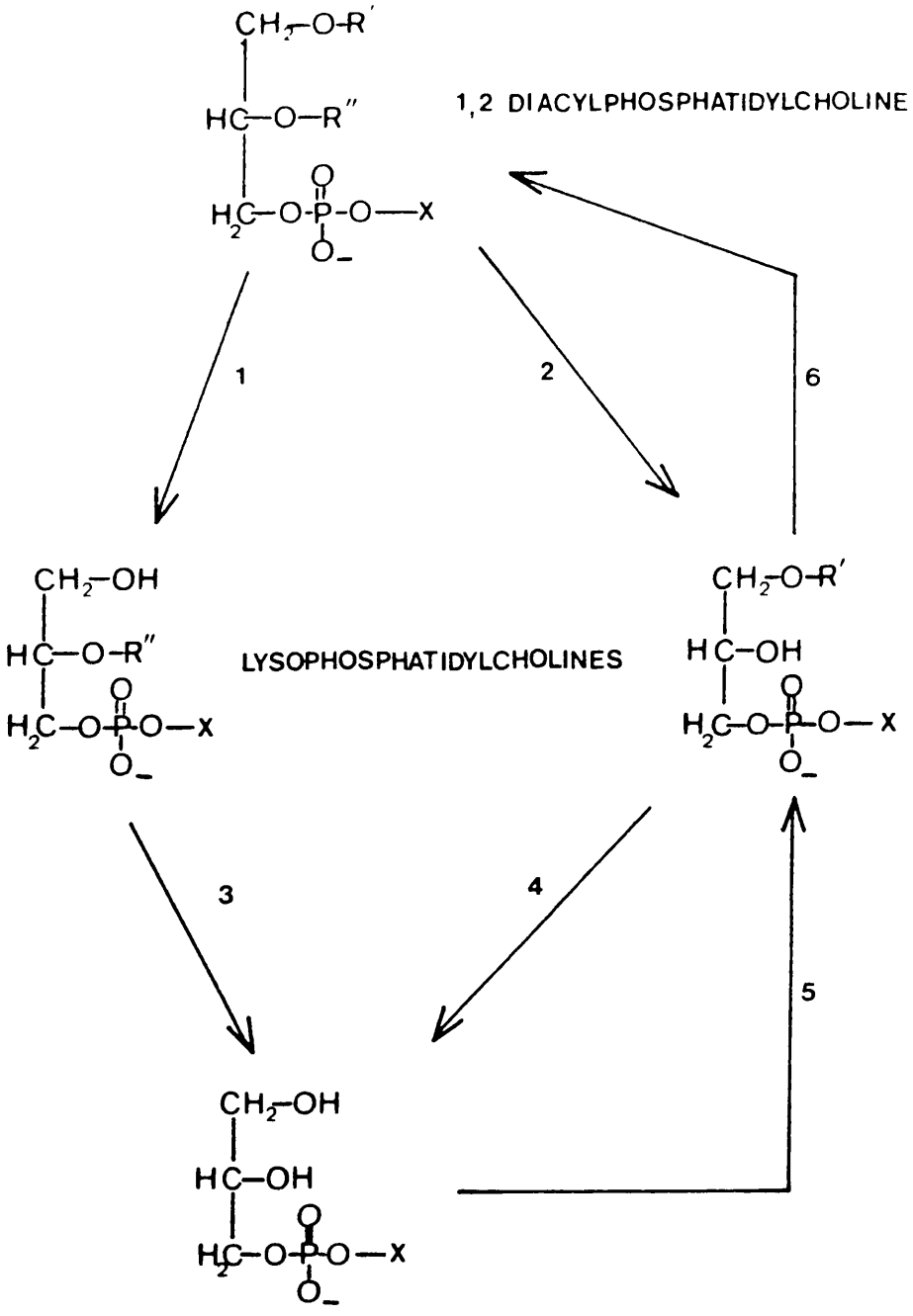


INOSITOL

R′ = Predominantly palmitic (C_{16}) & stearic (C_{18}) saturated fatty acids

R″ = Predominantly oleic (C_{18}) & linoleic (C_{18}) — mono & di-unsaturated & arachidonic — tetra-unsaturated fatty acids (C_{20})

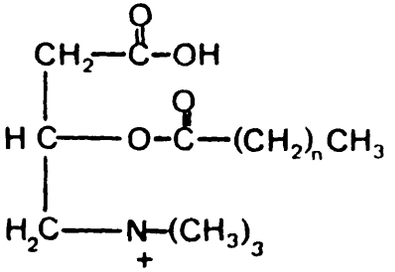
FIG 1. STRUCTURE OF MAJOR DIACYLPHOSPHOLIPIDS



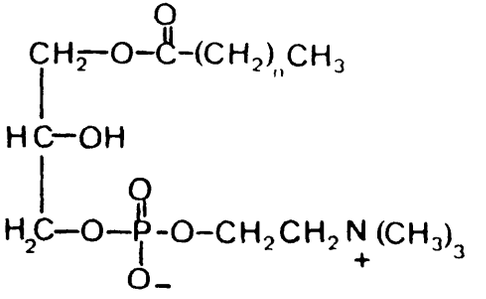
X = CHOLINE 1= PHOSPHOLIPASE A_1 2= PHOSPHOLIPASE A_2

3&4= LYSOPHOSPHOLIPASES 5&6= REACYLATION

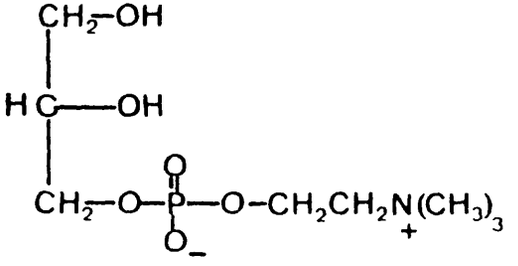
FIG 2. PATHWAYS INVOLVED IN THE METABOLISM OF PHOSPHOLIPIDS



PALMITOYL CARNITINE



PALMITOYL LYSOPHOSPHATIDYLCHOLINE



GLYCEROPHOSPHORYLCHOLINE

FIG 3. STRUCTURE OF LYSOPHOSPHATIDYLCHOLINE, PALMITOYL CARNITINE, AND GLYCEROPHOSPHORYL-CHOLINE.

METHODS

2 MEASUREMENT OF PHOSPHOLIPIDS AND LYSOPHOSPHOLIPIDS IN THE ISOLATED RAT HEART

2.1 Heart perfusion by the Langendorff method

Male Wistar rats (Bath University strain) of mass 250-300g were used throughout.

Hearts were removed, after killing the rat with a blow on the head, and placed in ice cold Krebs-Henseleit buffer of the following composition:

Krebs Henseleit buffer

	mM
NaCl	118.0
$NaHCO_3$	25.0
KCl	4.7
Glucose	11.6
KH_2PO_4	1.2
$MgSO_4.7H_2O$	1.2
$CaCl_2.6H_2O$	1.25

This composition was used throughout unless otherwise stated.

Hearts were perfused retrogradely through the aorta by the Langendorff method with Krebs-Henseleit buffer, saturated with 95% O_2 / 5% CO_2 to maintain a pH of 7.4, at 37°C at a constant flow rate of 10ml/min. Contractility was monitored isometrically with a

Dynamometer VFI transducer and perfusion pressure measured with a Washington PT400-5/N259 transducer. Perfusion pressure was typically 60-70mmHg. Hearts were perfused to waste for 5 minutes to remove blood elements before switching to a recirculating system of volume 12ml (FIG 4). This allowed continual reoxygenation of the recirculating buffer. Equilibration of the heart, as judged by steadiness of the contractility and perfusion pressure recordings, was normally achieved after 5 minutes of recirculation.

For coronary artery ligation experiments a thread was passed around the left descending coronary artery using a curved needle of 16mm length. The artery could then be ligated at the required time, an increase in perfusion pressure being considered indicative of successful ligation. On completion of the experiment the heart was removed from the cannula and an area of the free left ventricular wall, corresponding to the ischaemic area where the coronary artery had been ligated, excised. In control (sham ligated) hearts a tissue sample was taken from the anatomically corresponding area.

All samples for phospholipid determination were rapidly frozen with liquid nitrogen cooled Wollenberger tongs, and stored in liquid nitrogen prior to extraction of the phospholipids, in order to minimise post mortem changes. Care was taken to minimise the time taken for

excision of the samples, freezing being accomplished within 15-20 seconds of removal of the heart from the cannula.

2.2 Extraction of phospholipids

The extraction procedure is based on that of Shaikh and Downar (1981) and avoids the intrapreparative production of lysophospholipids from plasmalogens which can occur if acidic solvents are used (Shaikh and Downar 1980, Shaikh and Downar 1981). Plasmalogens are glycerophospholipids with an alkenyl group at the C-1 acyl position, this group being acid labile (Snyder 1969). Plasmalogens have been reported to comprise from 18% (Gloster and Harris 1969) to 40% (Fleischer, Klouwen and Brierley 1961, Mogelson, Wilson and Sobel 1980) of myocardial phospholipids and therefore an extraction procedure must be used that allows complete phospholipid recovery without plasmalogen breakdown. The procedure used is a modification of the Folch method of lipid extraction (Folch, Lees and Sloane-Stanley 1957) and has been shown to achieve almost complete phospholipid recovery (Shaikh and Downar 1981).

i) Method of extraction

a) Frozen tissue of mass 200mg was powdered in a liquid nitrogen cooled percussion mortar.

b) The powdered tissue was homogenised with a

Potter homogeniser in 5ml chloroform / methanol (2/1) containing 0.005% butylated hydroxytoluene (BHT) as an antioxidant.

c) The homogeniser tube was washed with a further 20ml of solvent and the washings added to the homogenate in a 50ml centrifuge tube.

d) After 5 minutes shaking 10ml of 0.9% saline were added to produce a biphasic mixture, followed by a further 5 minutes shaking.

e) Centrifuged at 2000rpm for 5 minutes in an MSE Chilspin at 4°C.

f) Lower layer removed by Pasteur pipette and retained.

g) Upper layer extracted a further 3 times with 15 ml chloroform / methanol / 0.58% sodium chloride (86/14/1). In each case the lower layer was retained and pooled in a round bottomed flask.

h) A final extraction of the upper layer was carried out with 15ml chloroform / methanol / 1M HCl (86/14/1), the lower layer being neutralised with aqueous ammonia prior to addition to the pooled extracts.

i) The combined extracts were taken to near dryness under nitrogen at 37°C, transferred to a 10ml tube with successive washings of the flask with chloroform / methanol / water (75/25/2). This was taken to dryness under nitrogen at 37°C and resuspended in 300µl of chloroform / methanol (2/1) for separation of

phospholipids by high pressure liquid chromatography.

ii) Extraction yields

The recovery of phospholipids through the extraction procedure was measured.

20µl of 0.7mg/ml lysophosphatidylcholine (LPC) (27nmol) was added to 25 ml of chloroform / methanol / BHT (2/1/0.005%) and taken through the extraction procedure before resuspending in 200µl of chloroform / methanol (2/1).

10µl aliquots were assayed for phosphate (section 2.4) as were aliquots containing 1.35 nmol LPC (equal to 100% yield of the extracted lipid).

n=4	Absorbance 660nm (-blank)
1.35nmol LPC	0.1395 +/- 0.011
1.35nmol LPC after extraction	0.1272 +/- 0.012

Extraction yield = 91%

2.3 Separation of phospholipids

i) Choice of method

The commonest techniques used for the separation of phospholipids are thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

a) TLC

TLC may require separation of the extract in two dimensions with different solvents to achieve sufficient resolution of the components (Snyder et.al. 1981). The lipids can then be visualised with iodine vapour (Snyder et.al. 1981) or an acid molybdate reagent (Dittmer and Lester 1964) and assayed for phosphate content (Shaikh and Palmer 1976).

b) HPLC

HPLC separation may be achieved with a silica gel (Chen and Kou 1982) or a cation exchange column (Gross and Sobel 1980). Separation on the silica gel column method of Chen and Kou (1982) depends on the presence of phosphoric acid in the solvent. Although this improves resolution the acidic nature of the solvent may lead to plasmalogen degradation. It also necessitates the use of an assay system for the phospholipids that would distinguish between inorganic and organic phosphate. A system without phosphoric acid is therefore preferable.

Using a cation exchange column lipids are retained on the column due to the presence of an amino functional group in the choline or ethanolamine portion of the lipid that is positively charged at neutral pH. This allows isocratic elution of the lipids with an organic solvent.

In the majority of HPLC systems the separated components are quantified by the absorbance peak area. This is not possible with the U.V. detection of phospho-

lipids as the U.V. absorbance around 200nm is due to the π-π^* transition of long chain unsaturated carbon-carbon double bonds present in the C-2 acyl group of most phospholipids (Jungalwala, Evans and McCluer 1976). This is dependent on the degree of unsaturation of the sample therefore in a tissue sample, where the degree of unsaturation is unknown, the absorbance does not directly correlate with the quantity of phospholipids and any saturated phospholipids would not be detected. Methods of quantifying the phospholipids by conversion into fluorescent dansyl (Chen, Kou and Chen 1981) or biphenyl-carbonyl derivatives (Jungalwala, Turel, Evans and McCluer 1975) have been reported but as this is only applicable to primary amino group containing phospholipids it would preclude the measurement of phosphatidylcholine and its lyso-derivative.

c) Method choice

It was decided to use an isocratic cation exchange HPLC system for separation of the phospholipids with collection of the eluates and assay for phosphate content.

ii) HPLC separation method

A Constametric LDC III chromatographic pump was used to pump a mobile phase of acetonitrile / methanol / water (55/35/10), degassed with helium before use, through a

Whatman Partisil 10 SCX 25cm x 4.6mm I.D. column at 1ml/min.

The eluate was passed to a Spectromonitor III U.V. detector for measurement of absorbance at 218nm. The relatively high wavelength used in comparison to that used by other workers (Gross and Sobel 1980) was used to reduce background absorption from the solvents in the mobile phase.

The order of elution was phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), the separation being complete in approximately 20 minutes (FIG 5). Retention times of the phospholipids were typically:-

	min
PE	3.5-4.5
LPE	5.0-6.5
PC	8.0-10.0
LPC	15.0-17.0

Variation in retention times was seen on different days, possibly due to temperature changes. Retention times were checked daily.

Phospholipids without amino groups are not retained on the column and should be eluted with the solvent front (Gross and Sobel 1980). This HPLC system is not able to separate PE from phosphatidylserine (PS). The concentration of PS is low in comparison to that of PE,

2% PS vs 26% PE (% of total phospholipids) in the human heart (Corr and Sobel 1981), 1.6% PS vs 36% PE in the rat myocardial sarcolemma (Tibbits et.al. 1981) and 2.1% PS vs 36% PE in rat ventricle mitochondria (Gloster and Harris 1970). Thus although the values determined for PE are likely to include some PS this is unlikely to be a major source of error. It does, of course, preclude the measurement of PS with this system.

iii) HPLC yields

20µl of 0.7mg/ml LPC (27nmol) was taken through the extraction procedure and the yield of the extraction procedure determined as previously described (section 2.2 ii).

20µl of the resuspended extract was injected onto the HPLC column and the eluate corresponding to LPC collected. This was dried down at 60°C, resuspended in 200µl chloroform / methanol (2/1) and 100µl assayed for phosphate content (section 2.4).

n=4	Absorbance at 660nm (-blank)
1.35nmol LPC	0.1395 +/- 0.011
1.35nmol LPC extracted + HPLC	0.1240 +/- 0.0009

Yield through extraction and HPLC = 89%

There was no significant loss during the HPLC separation as this yield is the same as that through the extraction

procedure.

A yield of 90% for extraction and HPLC separation was assumed for all further work.

2.4 Quantification of phospholipids

i) Choice of method

An assay of high sensitivity and accuracy was required to enable measurement of phosphate in the 0-2nmol range. The commonly used methods of Fiske and Subbarow (1925) and the modified Bartlett procedure (1959) do not allow such sensitivity.

An assay dependent on the formation of a complex between Zn^{2+}, molybdate and phosphate at pH 5 was investigated (Bencini, Wild and O'Donovan 1983). This complex does not need strongly acidic conditions, uses a single reagent with a shelf life of more than a year and is measured by absorbance at 350nm. It was found that acids suitable for use in the digestion of phospholipids such as perchloric, sulphuric and trichloroacetic all interfered with the assay such that very high absorbances were found with reagent blanks. Thus although this assay is suitable for inorganic phosphate it is not suitable for organic phosphate.

The assay used was based on that of Hess and Derr (1975). This uses the formation of a complex between malachite green and phosphomolybdate at low pH to produce

a coloured complex when phosphate is present.

ii) Phosphate assay method

Reagents

a) 0.045% malachite green hydrochloride (aq). Stored at room temperature.

b) 4.2% ammonium molybdate in 4M HCl.

c) 3 volumes of (a) plus 1 volume of (b) mixed and stirred for 30 minutes. This was filtered through a Whatman no. 1 filter paper prior to addition of 1/50th volume of 2% Tween 20.

Reagent (c) was made up freshly before each assay due to its relative instability, blank values increasing with age of the reagent (Hess and Derr 1975 and preliminary experiments). All glassware was acid washed to prevent contamination with detergent.

300µl of reagent (c) was added to 70µl of sample to be assayed, mixed and left at room temperature for 30 minutes for colour development. The absorbance at 660nm was measured in a dual beam spectrophotometer (Cecil CE 595) against a reagent blank.

iii) Phosphate standard curve

Standard curves were constructed to establish its linearity with respect to phosphate concentration.

Inorganic phosphate (KH_2PO_4) was used in varying

concentrations in a volume of 70µl as a standard (FIG 6).For concentrations 0.5-1nmol r=0.991.

Organic phosphate was obtained by digesting PC overnight at 80°C in 30µl 60% perchloric acid, cooling the tubes, and adding 40µl of distilled water prior to assay as for the inorganic phosphate (FIGS 6 + 7). Care was taken to work within the linear portion of the curve i.e. above 0.5nmol phosphate. For 0.5-1nmol r=0.9820. For phosphate up to 80nmol r=0.9963.

2.5 Protein assay

Phospholipid content of the heart was measured per unit protein.

The protein assay used was that of Read and Northcote (1981). It has advantages over the commonly used Lowry method (Lowry, Rosebrough, Farr and Randall 1951) as the reagent is stable at room temperature, incubation periods are not necessary and there is less interference from other chemicals.

Assay method

10mg of Brilliant Blue G was dissolved in 5ml of 95% ethanol. 10ml of concentrated orthophosphoric acid was added and made up to 100ml with distilled water. This was filtered twice before use to remove solid particles.

100µl of bovine serum albumin (BSA) at concentrations of 0-1mg/ml were added to 3ml of Brilliant

Blue reagent and the absorbance at 595nm read immediately against a reagent blank. A standard curve was always constructed in parallel to the sample assay although little variation in the standards was seen.

2.6 Protein content of the isolated rat heart

To enable calculation of phospholipid content per unit protein heart samples were prepared as follows.

Samples of approximately 200mg wet weight were taken from liquid nitrogen freeze clamped tissue, chopped finely and homogenised in 20ml of distilled water. Aliquots of this were assayed for protein content. For 5 hearts, protein content = 193 +/- 2.2 mg protein / g wet weight. This figure was used in all further work.

2.7 Phospholipid composition of the isolated rat heart after 10 minutes coronary artery ligation

Hearts were perfused to waste for 5 minutes, then in a recirculating system for 5 minutes prior to either sham ligation for 10 minutes (controls) or coronary artery ligation (CAL) for 10 minutes.

At the end of the 10 minute period of ligation tissue samples were taken from the ischaemic and non-ischaemic areas. Samples from corresponding areas were taken from the controls. Sample mass was determined (approx. 200mg) for calculation of protein content. Phospholipids were extracted, separated by HPLC and

quantified by phosphate assay.

2.8 Time course of changes in phospholipid composition during ischaemia in the isolated rat heart

Hearts were perfused as in section 2.7 prior to coronary artery ligation or sham ligation.

Samples were taken from the ischaemic region at periods from 0 to 30 minutes following ligation. Sham ligated controls at 15 and 30 minutes were carried out. Phospholipids were extracted, separated by HPLC and assayed by phosphate content.

2.9 Effect of perfusate ionic changes on phospholipid composition in the isolated rat heart

Hearts were perfused with different composition Krebs-Henseleit buffers to determine the effect of ionic changes on phospholipid composition.

The standard Krebs-Henseleit used had a calcium concentration of 1.25mM and a potassium concentration of 5.9mM. The following combinations were used, all other components of the buffer remaining the same.

a) 1.25mM Ca^{2+}, 5.9mM K^{+} -----standard

b) 1.25mM Ca^{2+}, 2.5mM K^{+}

c) 2.5mM Ca^{2+}, 5.9mM K^{+}

d) 2.5mM Ca^{2+}, 2.5mM K^{+}

Hearts were subjected to 20 minutes coronary artery

ligation and compared with sham ligated controls at time 0. Samples were taken from the ischaemic or anatomically corresponding area for determination of phospholipid composition.

3 EFFECT OF LYSOPHOSPHOLIPIDS AND RELATED COMPOUNDS IN THE ANAESTHETIZED CAT

Cats of mass 3-5 kg of either sex were anaesthetized with halothane and O_2 / N_2O (2/1) prior to injection of α- chloralose (80mg/kg) via the femoral vein. A tracheotomy was carried out to allow ventilation with room air at a stroke volume of 45cc and rate of 19 strokes/min with a Palmer respiration pump. This maintained arterial pO_2 around 100 mmHg, pCO_2 around 35 mmHg and pH at 7.3-7.4, values within the physiological range. Blood gases were regularly checked using an ABL blood gas laboratory. Body temperature was maintained at 36-38^oC with a heated blanket and monitored with an intragastric temperature probe. Blood pressure was measured in the left carotid artery by a pressure transducer linked to a Grass model 7D polygraph. Lead II E.C.G. was measured from limb leads.

A left thoracotomy was performed with the removal of one rib to allow suspension of the heart in a pericardial cradle. This allowed easy access to the left ventricular wall and left anterior descending coronary artery.

3.1 Infusion of lysophosphatidylcholine and palmitoyl-carnitine into the left ventricular wall of the anaesthetized cat

Infusions into the left ventricular wall were

carried out with a Perfusor ED2 infusion pump to allow accurate control of the infusion rate. A 27 gauge needle connected to the infusion syringe was used to puncture the ventricular wall to a depth of 2mm. Initial infusion was carried out with Evans Blue dye (0.2mg/ml in 0.9% saline) at 1ml/hr to visualise the area being perfused. The infusion rate was maintained at 1ml/hr with all solutions. On changing the infusion solution the needle remained *in situ* thus no variation was introduced by repositioning the needle.

The dye infusion was replaced with one of the following:

i) Lysophosphatidylcholine (LPC) at concentrations of 20-2000μM.

LPC was prepared by dissolving in chloroform / methanol (2/1) and drying under nitrogen before resuspending in 0.9% saline and sonicated for 5 minutes immediately before use.

ii) Palmitoylcarnitine (PAL) at concentrations of 20-1000μM prepared in the same manner as LPC. This was kept at 30°C to prevent the PAL forming an emulsion.

iii) Noradrenaline at a concentration of 10μM.

This was used as a further check on the positioning of the needle. In the pig noradrenaline has been shown to produce reproducible, reversible ventricular tachycardia (Podzuweit 1982) and thus if this is produced in the cat it demonstrates that the infusion

is penetrating the tissue.

3.2 Infusion of lysophosphatidylcholine, glycerophosphorylcholine, and palmitoylcarnitine into the coronary artery of the anaesthetized cat.

The cats were prepared surgically as previously. The left anterior descending coronary artery was cleared of surrounding tissue and a thread passed loosely around it.

Infusions into the coronary artery were carried out using a 34 gauge needle (Coopers Instruments) that was sufficiently flexible to allow a 90^{o} angle close to its tip without occluding the lumen. This allowed the insertion of the needle with the portion within the artery being parallel to the artery wall and therefore increased the probability of successful infusion.

Initial infusion was again carried out with Evans Blue dye (0.2mg/ml in 0.9% saline) to validate the needle position. If the needle had been accidently placed with the tip in the artery wall , rather than its lumen, or in the surrounding tissue, the area would rapidly become stained with the dye. No staining occurred if the needle was correctly placed.

To calculate the final blood concentrations of the infusate a coronary blood flow had to be assumed as there were no flow probes of a sufficiently small size available to enable direct measurement. A coronary blood

flow of 4ml/min was assumed. An average coronary blood flow of 120ml/min for a 60kg man (Gorlin and Herman 1978) would, if scaled down to a 3kg cat, give a value of 6ml/min. As the infusions were carried out slightly distal to the first branch of the artery the effective flow is likely to be less than this. Complete coronary artery bypass has been achieved in cats using a flow rate of 4ml/min (Snyder et.al. 1981). As this allowed maintenance of the animal without E.C.G. or haemodynamic alterations it is unlikely to be a gross underestimate of coronary flow.

Infusions were carried out as follows:

i) Lysophosphatidylcholine at final blood concentrations of 20μM-500μM.

ii) Palmitoylcarnitine at final blood concentrations of 10μM-75μM.

iii) Glycerophosphorylcholine at a final blood concentration of 400μM.

All infusions were carried out for 1 hour unless death occurred earlier.

Glycerophosphorylcholine was obtained as its cadmium chloride complex and the free glycerophosphorylcholine had to be prepared from this. An excess of solid silver carbonate was added to a saline solution of the complex. On shaking, the chloride precipitates as silver chloride and the cadmium as cadmium carbonate. Filtering produces

a solution of free glycerophosphorylcholine.

During infusions blood pressure and lead II E.C.G. were monitored. In i) and ii) tissue samples were taken at the completion of the experiment for the determination of phospholipase A_2 activity (section 5.0). Samples were taken from the left and right ventricular walls. In the case of hearts that had LPC or PAL infused into the coronary artery the left ventricular wall would be expected to receive the highest concentrations of the infused LPC or PAL, and the right ventricular wall the lowest. These areas correspond anatomically to the ischaemic and non-ischaemic regions of hearts that had undergone coronary artery ligation. Samples were taken from the same position in the wall in each experiment to minimise variation due to location.

3.3 Effect of coronary artery ligation on the production of arrhythmias and on temperature in the left ventricular wall of the anaesthetized cat

Cats were prepared surgically as previously. The left anterior descending coronary artery was ligated and the production of arrhythmias monitored. In some experiments the temperatures in the ischaemic and non-ischaemic zones were measured using electric thermometer probes (Light laboratories). The positioning of the probes was checked at the end of the experiment by

injecting Evans Blue dye i.v. The ischaemic area remained unstained.

Tissue samples were taken from ischaemic and non ischaemic regions for determination of phospholipase A_2 activity.

This enabled comparison of the effects of coronary artery ligation on the production and time course of arrhythmias, and on the temperature of the left ventricular wall with the effects of LPC, PAL and GPC (when infused into the coronary circulation) on the same parameters.

3.4 Effect of palmitoylcarnitine and streptokinase infusion into the coronary artery of the anaesthetized cat on the left ventricular wall temperature

Cats were prepared surgically as previously and the left anterior descending coronary artery cleared of surrounding tissue. Monitoring of the temperature of the left ventricular wall was carried out throughout.

PAL was infused at a final blood concentration of 20μM (assuming a blood flow of 4ml/min). Streptokinase (170U/min) was then infused to see if reversal of the effects of PAL on ventricular wall temperature could be obtained.

3.5 Effect of lysophosphatidylcholine infusion into the coronary artery of the anaesthetized cat on the left ventricular wall temperature

Cats were prepared surgically as previously.

Infusion of LPC into the left descending coronary artery, at a final blood concentration of 300μM, was carried out (assuming a coronary flow of 4ml/min) with simultaneous measurement of the left ventricular wall temperature. This enabled comparison with the effects of PAL.

3.6 Effect of lysophosphatidylcholine and palmitoylcarnitine on rat and rabbit platelet aggregation

The effect of LPC and PAL on rat and rabbit platelet aggregation was determined to see if this bore any correlation with their effects when infused into the coronary artery of the anaesthetized cat.

i) Preparation of platelet rich plasma

Rat blood (obtained by cardiac puncture) or rabbit blood (obtained by venepuncture) was immediately added to 3.2% sodium citrate (1 volume sodium citrate to 9 volumes blood) to prevent clotting. This was centrifuged at 1000rpm for 10 minutes to sediment the erythrocytes, producing a supernatant of platelet rich plasma (PRP). This was kept at room temperature and used

immediately.

ii) Measurement of platelet aggregation

200µl of PRP was pipetted into a small plastic tube and placed in an aggregometer, heated to 37°C, with stirring. After equilibration of 4-5 minutes additions of LPC or PAL were made in a small volume (less than 10µl) with a Hamilton syringe. LPC and PAL were dried down under nitrogen from chloroform / methanol (2/1) solutions, resuspended in distilled water and sonicated in a Soniclear bath immediately prior to use.

Aggregation in response to 50µg ADP (in 5µl) was used to test for platelet viability.

4 MEASUREMENT OF PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART

4.1 Assay choice

Phospholipase A_2 activity can be measured, like most enzymes, by assaying for substrate disappearance or product formation. However due to the low catalytic turnover of cellular phospholipases these enzymes are best assayed using a radiolabelled substrate. This allows the accurate detection of a small amount of substrate, and from thence, product. It also allows differentiation between the two fatty acids of the phospholipid and thus phospholipase A_1 and A_2 can be measured separately by

selective labelling.

Phospholipase A_2 activity was measured using phosphatidylethanolamine labelled with C^{14}-oleic acid in the 2-acyl position. PE was used as phospholipase A_2 appears to be more specific for this phospholipid (Weglicki, Waite, Sisson and Shohet 1971, Richards, Irvine and Dawson 1979, Wurl and Kunze 1985). This may be due to a different physical presentation of the phospholipid as it forms hexagonal arrays rather than the bilayers the majority of phospholipids form (Waite 1985), however this substrate preference has also been shown with endogenous substrates (Bjørnstad 1966).

4.2 Preparation of 1-acyl-2(1-C^{14}-oleoyl)glycero-3-phosphorylethanolamine

Based on the method of Blackwell, Duncombe, Flower, Parsons and Vane (1977).

i) Preparation of rat liver microsomes

Method of Warner and Lands (1961)

a) Tissue was rinsed in saline, homogenised in 3 volumes of 0.25M sucrose with a Potter homogeniser and centrifuged in a bench centrifuge at 2000g for 15 minutes to pellet nuclei and unbroken cells.

b) Pellet was resuspended in the same volume of 0.25M sucrose and recentrifuged at 2000g for 15 minutes.

The supernatant was combined with that of a).

c) The combined supernatant was centrifuged at 13,000g for 20 minutes at 4°C in an MSE 65 centrifuge for 90min to pellet the mitochondria and lysosomes.

d) The supernatant from c) was centrifuged at 78,000g in an MSE Superspeed 65 centrifuge to pellet the microsomes.

e) The pellet (microsomes) was resuspended in a volume of 0.25M sucrose equivalent to that of the original tissue volume (assuming 1g wet weight = 1ml).

Microsomes can be kept frozen for long periods of time without loss of activity (Warner and Lands 1961) but were prepared and frozen 24 hours before use.

ii) Synthesis of 1-acyl-2(1-C^{14}-oleoyl)glycero-3-phosphorylethanolamine

a) 250μCi of 1-C^{14} oleic acid (57mCi/mmol), 2mg carrier oleic acid and 20mg L-α-lysophosphatidylethanolamine were pooled in a conical flask and the organic solvents removed under nitrogen.

b) 150ml of 0.1M phosphate buffer, pH 7.5, was added and sonicated to disperse the lipids.

c) The following reagents were added:

200μmol ATP (pH 6.5 in water)

200μmol $MgCl_2$

2μmol Coenzyme A (sodium salt adjusted to pH

6.5 with sodium bicarbonate)

3ml of the suspension of rat liver microsomes in 0.25M sucrose

d) This was incubated at 37°C with shaking for 3 hours. At 30 and 120 minutes further equal amounts of ATP, $MgCl_2$, CoA and microsomes were added.

e) After 3 hours the reaction was terminated with 225ml of chloroform / methanol (2/1), shaken in a separating funnel and the lower layer decanted off.

f) The lower layer was dried under nitrogen and resuspended in a small volume of chloroform for subsequent separation of the labelled PE from oleic acid.

iii) Separation of 1-acyl-2(1-C^{14}-oleoyl)glycero-3-phosphorylethanolamine from C^{14} oleic acid

a) 2g of Florisil (Sigma activated magnesium silicate TLC grade) was suspended in chloroform and poured into a plastic column (volume 5ml) at 4°C.

b) The labelled mixture was applied to the column and the radioactivity of 5ml fractions, eluted from the column by chloroform , monitored by liquid scintillation counting (LKB 1215 Rackbeta with 5ml Optiphase scintillant). When the radioactivity in the eluted fractions approached zero the solvent was changed to methanol. The radioactivity of 5ml fractions eluted with methanol were likewise monitored (FIG 8).

Chloroform will elute unreacted oleic acid, methanol will elute the labelled PE. This was validated using TLC separation of unlabelled oleic acid and PE following their elution from a column and visualisation with iodine vapour.

c) The fractions eluted with methanol were pooled, dried down under nitrogen, and resuspended in 150ml of chloroform / methanol (2/1). 20µl samples were separated by TLC (in a solvent of chloroform / methanol / acetic acid (90/5/5)) with concomitant running of standards.

The following results were obtained:

	DPM
PE	38388 +/- 655
oleic acid	1187 +/- 88

The solution of labelled phospholipid in chloroform / methanol (2/1) was stored in sealed ampoules at -20°C until use.

4.3 Phospholipase A_2 assay

i) Assay method

a) 20µl chloroform / methanol (2/1) solution containing 1-acyl-2(1-C^{14}oleoyl)glycero-3-phosphoryl-ethanolamine (2µM labelled PE and total lipid concentration 146µM in the assay) was dried down under

nitrogen in a 1ml Eppendorf tube. The substrate was at saturating substrate concentration (preliminary experiments)

b) The lipid was resuspended in 50µl of 150mM Tris-/maleate pH 7 and sonicated for 30 seconds to disperse the lipid.

c) 50µl of 15mM $CaCl_2$ was added (final calcium concentration = 5mM)

d) 50µl of sample (heart homogenate, mitochondria or sarcolemma) for determination of phospholipase A_2 activity added. Unless stated otherwise the tissue sample was prepared in 150mM Tri/maleate pH 7 to produce a final buffer concentration of 100mM. Blanks without protein were carried out with every assay to correct for non enzymic degradation of the substrate.

e) Incubation at 37°C for 45 minutes.

f) Reaction stopped by addition of 188µl chloroform, 375µl methanol, vortexed, and a further 188µl chloroform and 188µl water added. This was vortexed and centrifuged for 4 minutes in an Eppendorf 5412 centrifuge.

g) The top layer and protein interface were removed with a Pasteur pipette and the lower layer dried under nitrogen.

h) Lower layer resuspended in 50µl chloroform/ methanol (2/1).

i) 20µl was applied to TLC plates (5x20cm) of silica gel Type G particle size 10-40µm spread to 250µm thickness. The plates were run in a solvent of chloroform

/ methanol / acetic acid (90/5/5) for 30 minutes, dried in air and the lipids visualised by exposure to iodine vapour.

j) The portions corresponding to the oleic acid- as evidenced by the concurrent running of a standard- were scraped off and counted by liquid scintillation counting.

Blanks with no protein were taken through the incubation procedure, extraction and TLC separation, and any radioactivity in the oleic acid portion subtracted from the experimental values. Protein content of the tissue samples was determined to calculate the phospholipase activity per mg protein per hour.

ii) Thin layer chromatography of reaction products

The solvent system used enabled separation of the oleic acid, which ran near the solvent front, from the labelled PE, which remained at the origin. The lyso derivative of PE also remains at the origin therefore should the substrate be acted upon by a phospholipase A_1 the radioactive product, lyso-PE, would not be measured. Likewise, the products of phospholipase C activity would not be measured as diolein,a diacylglycerol, was shown to migrate with the solvent front. Phosphatidic acid, a phosphorylated metabolite of diacylglycerol, can be distinguished from oleic acid.

Typical Rf values

oleic acid	0.8
diacylglycerol	1.0
phosphatidic acid	0.36
PE	0-0.1
LPE	0-0.1

iii) Yields of C^{14} oleic acid through the extraction procedure

a) 10µl of C^{14} oleic acid was added to 150µl 100mM Tris/maleate pH 7.5

b) 10µl of C^{14} oleic acid was added to 100µl 100mM Tris/maleate pH 7.5 and 50µl heart homogenate. Final concentration of Tris/maleate = 100mM.

These solutions were put through the lipid extraction procedure. After centrifugation to separate the two phases the lower, chloroform phase was counted by liquid scintillation counting. In an additional experiment samples of the same composition as b) were extracted, dried down under nitrogen, resuspended in chloroform / methanol (2/1) and counted [c)].

Yields

Counts in 10µl oleic acid solution = 42912 d.p.m.

n=3

a) 77.3 +/- 2.8 %

b) 82.8 +/- 2.26 %

c) 76.4 +/- 3.9 %

The yields between the three groups were not significantly different. There is therefore no loss of oleic acid due to the presence of protein in the assay or due to the final drying down and resuspension of the extract.

For all samples:- yield = 78.8%. This yield was assumed for all further experiments.

4.4 Characterisation of phospholipase A_2 in the homogenate of the isolated rat heart

i) Protein dependence

Male Wistar rats (250-300g) Bath University strain were used throughout.

A rat heart was perfused retrogradely through the aorta by the Langendorff method for 5 minutes with 0.9% saline to remove blood elements. The heart was then homogenised with an Ultra Turrax homogeniser in 6ml of 150mM Tris/maleate pH 7. Phospholipase A_2 activity was determined with 3-5 volumes of homogenate from 0-50µl, the volume being made up to 50µl with the buffer. Boiled controls were carried out to demonstrate the enzymic nature of the assay.

ii) Calcium dependence

The activity of phospholipase A_2 was determined with concentrations of calcium from 0 to 5 mM.

50µl aliquots of heart homogenates in 150mM Tris/maleate pH 7 were incubated with 50µl of buffer and varying volumes of 15mM $CaCl_2$ from 0 to 50µl, the volume being made up with distilled water. Comparison was made with aliquots incubated with 10mM EDTA (disodium salt) replacing the calcium.

iii) Time dependence

To determine the degree of linearity of the reaction with time 50µl aliquots of heart homogenate were incubated for 15, 30 and 45 minutes before stopping the reaction. To correct for spontaneous, non-enzymic, release of C^{14}-oleic acid controls with no homogenate present were incubated for 15 and 60 minutes. Spontaneous breakdown of the labelled substrate was assumed to be linear with time and thus control values for the other times could be calculated. These control values enabled calculation of C^{14}-oleic acid release at the various times due to enzymic activity only.

iv) Loss of activity on freezing

All samples, unless stated otherwise, were assayed freshly.

Samples of tissue from the in vivo cat work had to be stored for up to 3 months before assay and therefore the effect of freezing on phospholipase A_2 had to be determined.

a) Samples previously assayed freshly that had been kept frozen at -20°C were reassayed to determine any change in phospholipase A_2 activity. These had been frozen for approximately 5 months.

b) A time course of activity loss was carried out. Hearts were homogenised in 150mM Tris/maleate pH 7 and separated into 5 portions, one of which was assayed

freshly. The others were frozen and assayed on subsequent days to calculate the change in activity over 12 days.

4.5 Effect of coronary artery ligation on phospholipase A_2 activity in the ischaemic and non-ischaemic areas of the isolated rat heart

Hearts were perfused as in section 2.7. Hearts were removed at 5 or 20 minutes post ligation and phospholipase A_2 activity determined in a homogenate of the ischaemic and non-ischaemic areas. Heart tissue was homogenised in approximately 1ml buffer per 150mg wet weight. Regions corresponding anatomically to these were taken from the sham ligated hearts.

A pH profile of activity was determined, the tissue being homogenised in 0.9% saline on ice and 50µl aliquots immediately added to 50µl 15mM $CaCl_2$ and 50µl of one of the following:

pH	Buffer
4	0.3M Sodium acetate
5	0.3M Sodium acetate
6	0.3M Tris/maleate
7	0.3M Tris/maleate
8	0.3M Tris/HCl
9	0.3M Tris/HCl

The final buffer concentration in the assay was 100mM and the pH that of the buffer used,i.e. dilution of

the buffer did not affect the resulting pH.

4.6 Mitochondrial phospholipase A_2

i) Preparation of mitochondria

a) Isolation of mitochondria

Heart tissue was placed in 250mM sucrose / 10mM TrisHCl pH 7.6 and chopped finely (1g wet weight tissue to 5 ml buffer). This was homogenised with the Potter homogeniser (2 passes of the pestle at mark 3) and centrifuged at 2000rpm for 5 minutes (MSE Chilspin) at 4°C to pellet the nuclei and whole cells. The supernatant was centrifuged at full speed in an Eppendorf 5412 centrifuge at 4°C for 8 minutes.

The pellet was resuspended in 250mM sucrose / 10mM TrisHCl pH 7.6 (approx. 1ml).

b) Mitochondrial integrity

Mitochondrial integrity was measured by measuring the respiratory control index (RCI).

The following were pipetted into the chamber of a Clark oxygen electrode:

	Volume(ml)	Concn.(mM)	Final Conc.(mM)
sucrose	1.5	667	200
Tris/HCl	1.125	40	10
KCl	0.64	315	40
K_3PO_4	0.64	7.8	1
mitochondrial	suspension 0.5ml		

before addition of mitochondrial suspension.

Oxygen was bubbled through to saturate the solution. After equilibration of the contents in the closed chamber 100µl of 85mM sodium succinate was added through the injection port (final concentration = 1.7mM) and oxygen consumption recorded with time (state 4 respiration).

50µl of 33mM ADP (pH 6.6) was added. Oxygen consumption of intact mitochondria increases on addition of ADP (state 3 respiration) and enables calculation of the respiratory control index.

$$RCI = \frac{\text{state 3 respiration rate}}{\text{state 4 respiration rate}}$$

Mitochondria prepared as above typically had an RCI of 2.5-3.7. As a reasonable degree of coupling exists the majority of the mitochondria are likely to be intact.

ii) Mitochondrial phospholipase A_2: pH dependency

Mitochondria were prepared from a heart sham ligated for 5 minutes. The pH dependency of the phospholipase A_2 was determined by incubating 50µl aliquots of mitochondrial suspensions with 50µl of 15mM $CaCl_2$ and 50µl of a 300mM buffer (pH 4-9). The pH 7.6 10mM Tris/HCl buffer of the mitochondrial suspension did not affect the final pH of the assay.

iii) Mitochondrial phospholipase A_2: calcium dependency

The activity of phospholipase A_2 was determined with concentrations of calcium from 0 to 10mM. 50µl aliquots of mitochondrial suspension from a heart sham ligated for 5 minutes were incubated with 50µl of 300mM Tris/maleate pH 7 and varying volumes of 30mM $CaCl_2$ from 0 to 50µl, the volume being made up with distilled water. Comparison was made with aliquots incubated with 10mM EDTA (disodium salt) replacing the calcium.

4.7 Effect of coronary artery ligation on the activity of mitochondrial phospholipase A_2 in the ischaemic and non-ischaemic regions of the isolated rat heart

Hearts were perfused as in section 2.7. Mitochondria were prepared from the ischaemic and non-ischaemic areas 20 minutes following coronary artery ligation.A pH profile of activity was determined with 50µl aliquots of the mitochondrial suspension being incubated with 50µl of 15mM $CaCl_2$ and 50µl of a 300mM buffer (pH 4-9).

4.8 Sarcolemmal phospholipase A_2

i) Preparation of sarcolemma

a) Isolation of sarcolemma

A crude sarcolemmal preparation was made by the method of Pang and Weglicki (1977).

Ventricular tissue was excised and weighed before homogenising with a Potter homogeniser in 10 volumes of 0.6M KCl / 250mM sucrose / 10mM imidazole pH 7. This was further homogenised with an Ultra Turrax homogeniser for 5 seconds. After centrifugation at 1600g for 20 minutes the pellet was washed twice with the same buffer, the supernatant being discarded in each case. The pellet was resuspended in 250mM sucrose / 10mM imidazole pH 7, centrifuged as before and resuspended in this buffer to remove the excess KCl.

b) Estimation of sarcolemmal enrichment

To estimate the degree of purification of sarcolemma achieved the content of sialic acid was measured as by Takahashi and Kako (1984) using N-acetyl neuraminic acid as a standard.

Sialic acid assay

Based on that of Warren (1959 and 1959a).

Reagents

1) 0.2M sodium periodate in 9M H_2SO_4

2) 10% sodium arsenite in 0.5M sodium sulphate / 0.1M H_2SO_4

3) 0.6% thiobarbituric acid in 0.5M sodium sulphate

These solutions are stable for a minimum of one month at room temperature.

Procedure

Standard calibration

i) 0 to 0.05μmoles of N-acetylneuraminic acid standard in a volume of 0.2ml was added to 0.1ml of reagent 1).

ii) This was shaken and left to stand at room temperature for 20 minutes.

iii) 1ml of reagent 2) was added and shaken until the yellow colour disappeared.

iv) 3ml of reagent 3) was added and shaken. The tubes were loosely capped and heated in a boiling water bath for 15 minutes before cooling in cold water for 5 minutes.

v) 1ml of the solution was added to 1ml of cyclohexanone, shaken thoroughly and centrifuged at 2000rpm for 5 minutes.

vi) The upper layer was pipetted off and the absorbance at 549nm read against a reagent blank (FIG 9).

Tissue samples (heart homogenate or isolated sarcolemma) were heated at 80°C for 1 hour in 0.1M H_2SO_4 (3 volumes sample to 1 volume acid) to release the tissue sialic acid without degradation. This allows their subsequent assay.

2-deoxyribose in tissue samples has an absorption maximum at 532nm and thus contributes to the absorbance measured at 549nm. This absorbance must be accounted for to allow measurement of the absorbance at 549nm due to sialic acid only.

Calculation of 2-deoxyribose contribution to absorbance at 549nm

For standards of N-acetylneuraminic acid:

$$Ab\ 549 / Ab\ 532 = 2.47 +/- 0.104$$

whilst for tissue samples assayed concurrently:

$$Ab\ 549 / Ab\ 532 = 0.996 +/- 0.06$$

This demonstrates absorbance at 532nm by 2-deoxyribose in the tissue samples. From Warren (1959) it can be seen that for N-acetylneuraminic acid and 2-deoxyribose this ratio is 2.26 and 0.3387 respectively.

If Ab 549 due to N-acetylneuraminic acid = x

and Ab 549 due to 2-deoxyribose = y

Ab 549 total = x + y, Ab 532 total = x/2.26 + y/0.3387

then 0.442x + 2.952y = Ab 532 total.

From the tissue samples assayed-

$$Ab\ 549\ total / Ab\ 532\ total = 0.996$$

thus $0.442x + 2.952y = Ab\ 549 / 0.996$

$0.440x + 2.94y = Ab\ 549\ total$

$0.440x + 2.94y = x + y$

$x = 3.464y$

From this the contribution of 2-deoxyribose and sialic acids to the absorbance at 549nm can be calculated and therefore the sialic acid concentration of the samples.

Using this method the degree of enrichment of 4 preparations of sarcolemma from the respective homogenates was calculated. The ratio of sialic acid in

the sarcolemma with that in the homogenate = 3.6 +/- 0.79 n=4. Sarcolemma = 160.9 +/- 26.3 nmol/mg protein and homogenate = 48.5 +/- 8.7 nmol/mg protein.

Calculations of the degree of purification of sarcolemma were carried out using hearts that had not undergone coronary artery ligation and yields in ischaemic tissue were assumed to be similar. This was assumed because of reports of increased sialic acid contents of ischaemic tissue (Takahasi and Kako 1984) which would lead to erroneous calculations of purification if this occured in the isolated rat heart.

The purification factor of 3.6 achieved compared with a ratio of 3.11 for crude sarcolemma using Na^+ / K^+ ATPase as a marker enzyme (Pang and Weglicki 1977) and 3.19, also for crude sarcolemma (Franson, Pang, Towle and Weglicki 1978). Much higher degrees of purification have been reported, from 7 fold (Pang and Weglicki 1977), 12 fold (Takahashi and Kako 1983), to 34 fold (Tibbits et.al. 1981). These preparations involved the use of sucrose density gradients for further purification of the crude sarcolemma. The crude sarcolemma were thought to be sufficient for the purposes of these experiments.

ii) Calcium dependence of sarcolemmal phospholipase A_2

Sarcolemmal preparations were made from hearts perfused for 10 minutes with Krebs-Henseleit by the Langendorff method.

50µl aliquots were assayed at calcium concentrations from 0 to 5mM using varying volumes of 15mM $CaCl_2$ and incubating with 50µl 300mM Tris/maleate pH 7. Comparison was made with aliquots incubated with 10mM EDTA (disodium salt) replacing the calcium.

4.9 Effect of coronary artery ligation on phospholipase A_2 activity in the sarcolemma of the ischaemic and non-ischaemic areas of the isolated rat heart

Hearts were perfused as in section 2.7. Sarcolemma were prepared from the ischaemic and non-ischaemic regions 20 minutes following coronary artery ligation, and resuspended in 1ml 250mM sucrose / 10mM imidazole pH 7. A pH profile of phospholipase A_2 activity from pH 4-9 was constructed with 50µl aliquots being incubated with 50µl of 15mM $CaCl_2$ and 50µl of a 300mM buffer pH 4-9.

4.10 Effect of lysophosphatidylcholine and palmitoyl-carnitine on phospholipase A_2 activity in homogenates of the isolated rat heart

The effects of LPC and PAL on phospholipase A_2 activity were determined.

Hearts perfused with Krebs-Henseleit buffer to remove blood elements were homogenised in 150mM Tris/maleate pH 7. 50µl aliquots were assayed with varying concentrations of LPC or PAL, 50µl of 15mM $CaCl_2$, and 50µl 150mM Tris/maleate pH 7. The LPC and PAL were

initially in solution in chloroform / methanol (2/1) and were dried down under nitrogen in the assay tube with the radiolabelled substrate prior to resuspension and sonication. Concentrations of LPC up to 400μM and of PAL up to 1000μM were used.

4.11 Effect of mepacrine on phospholipase A_2 activity in homogenates of the isolated rat heart

Homogenates of rat hearts prepared as in 4.10 were assayed for phospholipase A_2 activity with 50μl of 15mM $CaCl_2$ and 50μl of 150mM Tris/maleate pH 7 added to 50μl aliquots of the homogenate. Mepacrine, at a maximum volume of 30μl was added to give a final concentration of 0-50μM. Controls with 30μl distilled water added were run to control for volume addition.

4.12 Effect of lysophosphatidylcholine, palmitoyl-carnitine and mepacrine on ADP induced platelet aggregation

The effect of LPC, PAL and mepacrine on ADP induced platelet aggregation was determined to see if this correlates with their effect on phospholipase A_2 activity.

Platelet rich plasma was prepared from rabbit blood. 200μl aliquots were placed in plastic tubes in a platelet aggregometer at 37°C with stirring. After 4-5 minutes equilibration LPC, PAL or mepacrine were added in the

required concentration in a maximum volume of 10µl, followed 3.5 minutes later by 50µg ADP (in 5µl).

The following concentrations were used to determine their effects on platelet aggregation induced by 50µg ADP.

LPC	0-500µM
PAL	0-1000µM
mepacrine	0-50µM

5 PHOSPHOLIPASE A_2 ACTIVITY IN HEART TISSUE SAMPLES FROM THE ANAESTHETIZED CAT

Samples were taken from the left and right ventricular walls of hearts treated as follows (sections 3.2 and 3.3).

i) Infused with LPC at final blood concentrations of 20-500µM via the left anterior descending coronary artery.

ii) Infused with PAL at final blood concentrations of 10-75µM via the left anterior descending coronary artery.

iii) Subjected to coronary artery ligation

In all cases samples were taken 1 hour after coronary artery ligation or initiation of infusion unless death occured prior to this, in which case samples were taken as rapidly as possible after death. Animals remaining alive after 1 hour were killed with saturated

KCl i.v. and tissue samples then taken.

Samples were stored at -20°C until assay. All phospholipase A_2 assays were carried out at pH 7 in the presence of 100mM Tris/maleate and 5mM $CaCl_2$.

6 EFFECT OF REPERFUSION OF THE ISCHAEMIC ISOLATED RAT HEART ON PHOSPHOLIPASE A_2 ACTIVITY

Hearts were perfused with Krebs-Henseleit buffer for 5 minutes to waste and 5 minutes in a recirculating system before ligation of the left coronary artery.

Ligation was achieved by passing both ends of the thread around the artery through a narrow bore polyethylene tube. This could be pulled against tne heart to occlude the artery and allowed subsequent reperfusion by releasing the tubing from the heart.

6.1 Effect of 10 minutes occlusion and 1 minute reperfusion on phospholipase A_2 activity in the isolated rat heart

Hearts were perfused as above (section 6) and the coronary artery occluded for 10 minutes prior to 1 minute reperfusion. Tissue samples were taken from the ischaemic and non-ischaemic regions for determination of phospholipase A_2 activity in tissue homogenates at pH 7 with 100mM Tris/maleate, 5mM $CaCl_2$.

6.2 Effect of a lower K^+ concentration Krebs-Henseleit buffer on phospholipase A_2 activity in the isolated rat heart following 10 minutes occlusion and 1 minute reperfusion

As no reperfusion arrhythmias were seen in section

6.1 the composition of the buffer was changed to one with a lower K^+ concentration, 3.2mM as opposed to 5.9mM in the standard Krebs-Henseleit. This makes the hearts more susceptible to reperfusion arrhythmias and thus the possible effect on the phospholipase A_2 activity can be determined.

Hearts underwent 10 minutes coronary artery ligation and 1 minute reperfusion prior to excision of tissue samples to determine phospholipase A_2 activity in the ischaemic and non-ischaemic areas.

6.3 Effect of the 3.2mM K^+ Krebs-Henseleit on phospholipase A_2 activity in the isolated rat heart during ischaemia

Controls were carried out for the 3.2mM K^+ buffer to ensure that any change in phospholipase A_2 activity in section 6.2 was due to reperfusion and would not occur after 10 minutes ischaemia.

Hearts were perfused as in section 6. Samples of the ischaemic and non-ischaemic areas were taken after 10 minutes coronary artery ligation for determination of phospholipase A_2 activity. Sham ligated controls were also carried out.

6.4 Measurement of the effects of eluted perfusate from reperfused hearts on phospholipase A_2 activity

As reperfusion caused a change in phospholipase A_2

activity it is possible that something in the eluted perfusate is capable of eliciting this effect.

i) Effect of perfusate on phospholipase A_2 activity

Hearts were perfused in the recirculating system with the 3.2mM K^+ Krebs-Henseleit and the coronary artery ligated for 10 minutes prior to 1 minute reperfusion. Samples of perfusate from the heart were collected just before and after reperfusion, kept on ice and used within 1 hour. 30µl of the perfusate was added to the assay of phospholipase A_2 in a homogenate of a sham ligated heart.

ii) Effect of lipid extract of reperfusion perfusate on phospholipase A_2 activity

a) Extraction method

A lipid extract of the perfusate collected from a reperfused heart was made as follows:-

6 x 300µl samples of perfusate added to 6 x 750µl chloroform / methanol / BHT (2/1/0.005%), shaken, centrifuged at full speed in an Eppendorf centrifuge for 5 minutes at 4°C and the lower layer retained. 3 further extractions of the upper layer were carried out with 450µl chloroform / methanol / 0.58% NaCl (86/14/1), the lower layers being retained and pooled. A final extraction was carried out with 450µl chloroform / methanol / 1M HCl (86/14/1) and the lower layer neutralised with aqueous ammonia before addition to the pooled extracts. These were dried under nitrogen and

resuspended in 1800µl chloroform / methanol (2/1). The concentration of any lipids in the lipid extract was therefore the same as in the perfusate.

Volumes of the extract from 30 to 90 µl were dried down under nitrogen with the labelled substrate for a subsequent phospholipase A_2 assay of a whole heart homogenate from a heart sham ligated for 10 minutes.

b) TLC of the lipid extract

Aliquots of lipid extract were spotted onto silica gel TLC plates of 250µm thickness and run in a system of chloroform / acetone / methanol / acetic acid / water (6/8/2/2/1) which allows separation of free fatty acids, PE, PC, LPC and LPE (Sobel, Corr, Robison, Goldstein, Witkowski and Klein 1978). After drying in air the lipids were visualised with iodine vapour. Standards of oleic acid and L-α- lysophosphatidylcholine were also run.

6.5 Effect of a free radical scavenger on phospholipase A_2 activity during reperfusion of the isolated rat heart

Changes in phospholipase A_2 activity during reperfusion may be due to the production of free radicals which cause lipid peroxidation and membrane damage (FIG 10). The effect of reduced glutathione, a cellular antioxidant, on the changes in phospholipase A_2 during reperfusion was determined.

1mM reduced glutathione was included in the 3.2mM K^+

Krebs-Henseleit buffer. The hearts were ligated for 10 minutes and reperfused for 1 minute prior to determination of the phospholipase A_2 activity in homogenates of the ischaemic and non-ischaemic areas.

6.6 Effect of a constant pressure head system on phospholipase A_2 activity during reperfusion of the isolated rat heart

All experiments have been done under constant flow (10ml/min). Thus on occlusion of the coronary artery perfusion of the non-ischaemic area would rise and then fall again on reperfusion. It is possible that changes in phospholipase A_2 activity are secondary to these flow changes. A constant head system was used to produce ischaemia and reperfusion where there should be no flow changes in the non-ischaemic area.

Hearts were perfused at a constant pressure head of 100cm water. During 10 minutes coronary artery occlusion coronary flow was 63 +/- 3.7% of that before occlusion (reduced from a mean of 10.9 ml/min to 6.89 ml/min n=4). After 1 minute reperfusion phospholipase A_2 activity was assayed in the ischaemic and non-ischaemic areas.

6.7 Effect of flow rate changes on phospholipase A_2 activity in the isolated rat heart

During coronary artery occlusion the non-ischaemic area is relatively overperfused. Assuming the decrease in

coronary flow seen on occlusion in a constant pressure system is typical, an increase of flow from 10ml/min to 15ml/min in a constant flow system would simulate, in a whole heart, the degree of overperfusion seen in the non-ischaemic area during coronary artery occlusion.

The effect of flow changes was determined as follows:

a) Hearts perfused for 10 minutes at 15ml/min

b) Hearts perfused for 10 minutes at 15ml/min and 1 minute at 10ml/min.

These simulate the flow changes in the non-ischaemic area in a) ischaemia and b) ischaemia + reperfusion

Global ischaemia was used to simulate, in a whole heart, the flow changes that occur in the ischaemic area of a heart with the coronary artery occluded as follows:

a) no flow for 10 minutes

b) no flow for 10 minutes and 1 min at 10ml/min.

These simulate the flow changes in the ischaemic area in a) ischaemia and b) ischaemia + reperfusion.

Phospholipase A_2 activity was then assayed in homogenates of the tissue.

6.8 Effect of vasodilators on phospholipase A_2 activity in the isolated rat heart on high flow rate perfusion

Changes in phospholipase A_2 activity which were seen on increasing flow may be due to the increased perfusion

pressure. The effect of two vasodilators, which should reduce the pressure in the vessels by causing dilatation, on phospholipase A_2 activity was determined.

i) Effect of adenosine

a) 10^{-5}M adenosine in a recirculating system

Hearts were perfused with 3.2mM K^+ Krebs-Henseleit containing 10^{-5}M adenosine for 10 minutes at 15ml/min in a recirculating system. The tissue was then homogenised and assayed for phospholipase A_2 activity.

b) 10^{-5}M and 10^{-6}M adenosine in a non-recirculating system

Adenosine is stable in Krebs-Henseleit (preliminary experiments) but has a short half life in blood (Klabunde 1983) and is rapidly taken up and metabolised by tissue (Jacob and Berne 1960, review Berne 1980). Therefore in the recirculating system, where the buffer containing adenosine has been passing through the tissue for 5 minutes before elevation of the flow rate to 15ml/min, it is likely that all the adenosine has been metabolised by then. A non-recirculating system was used for comparison so that all the Krebs-Henseleit passing through the heart contains adenosine that has not been metabolised.

Hearts were perfused with 3.2mM K^+ Krebs-Henseleit containing 10^{-5}M or 10^{-6}M adenosine in a non-recirculating system at 15ml/min for 10 minutes. The tissue was then homogenised and assayed for phospholipase

A_2 activity.

ii) Effect of sodium nitroprusside

Hearts were perfused with 3.2mM K^+ Krebs-Henseleit containing 10^{-4}M sodium nitroprusside at 15ml/min in a recirculating system. The tissue was then homogenised and assayed for phospholipase A_2 activity.

MATERIALS

CHEMICALS

Acetic acid (glacial)	Fisons
N-acetylneuraminic acid	Sigma
Adenosine diphosphate	"
Adenosine triphosphate	"
Brilliant Blue G	"
Cadmium chloride	BDH
α- chloralose	"
Coenzyme A (sodium salt)	Sigma
Florisil (TLC grade)	"
L-α- glycerphosphorylcholine (grade 1, cadmium chloride complex)	"
Imidazole (grade 1)	"
Iodine crystals	BDH
L-α- Lysophosphatidylcholine (Type 1 from egg yolk)	Sigma
L-α- Lysophosphatidylethanolamine (Type 1 from egg yolk)	"
1-C^{14}-oleic acid	Amersham Int.
Oleic acid (sodium salt)	Sigma
Orthophosphoric acid	BDH
DL-α-palmitoylcarnitine	Sigma
Perchloric acid (60%)	Fisons
L-α- phosphatidylcholine (from bovine liver)	Sigma
L-α- phosphatidylethanolamine (from bovine liver)	Sigma

Silica gel type G (particle size 10-40um)	"
Silver carbonate	BDH
Sodium arsenite (analar)	"
Sodium periodate	BDH
Streptokinase (β-haemolytic streptococcus)	Sigma
2-thiobarbituric acid	"
Tris base	"

SOLVENTS

Acetone	Fisons
Acetonitrile	"
Chloroform	"
Ethanol (95%)	"
Cyclohexanone (analar)	BDH
Methanol	Fisons

STATISTICS

Significance was assessed by the group or paired t-test as appropriate and a p value of 0.05 or less taken as significant.

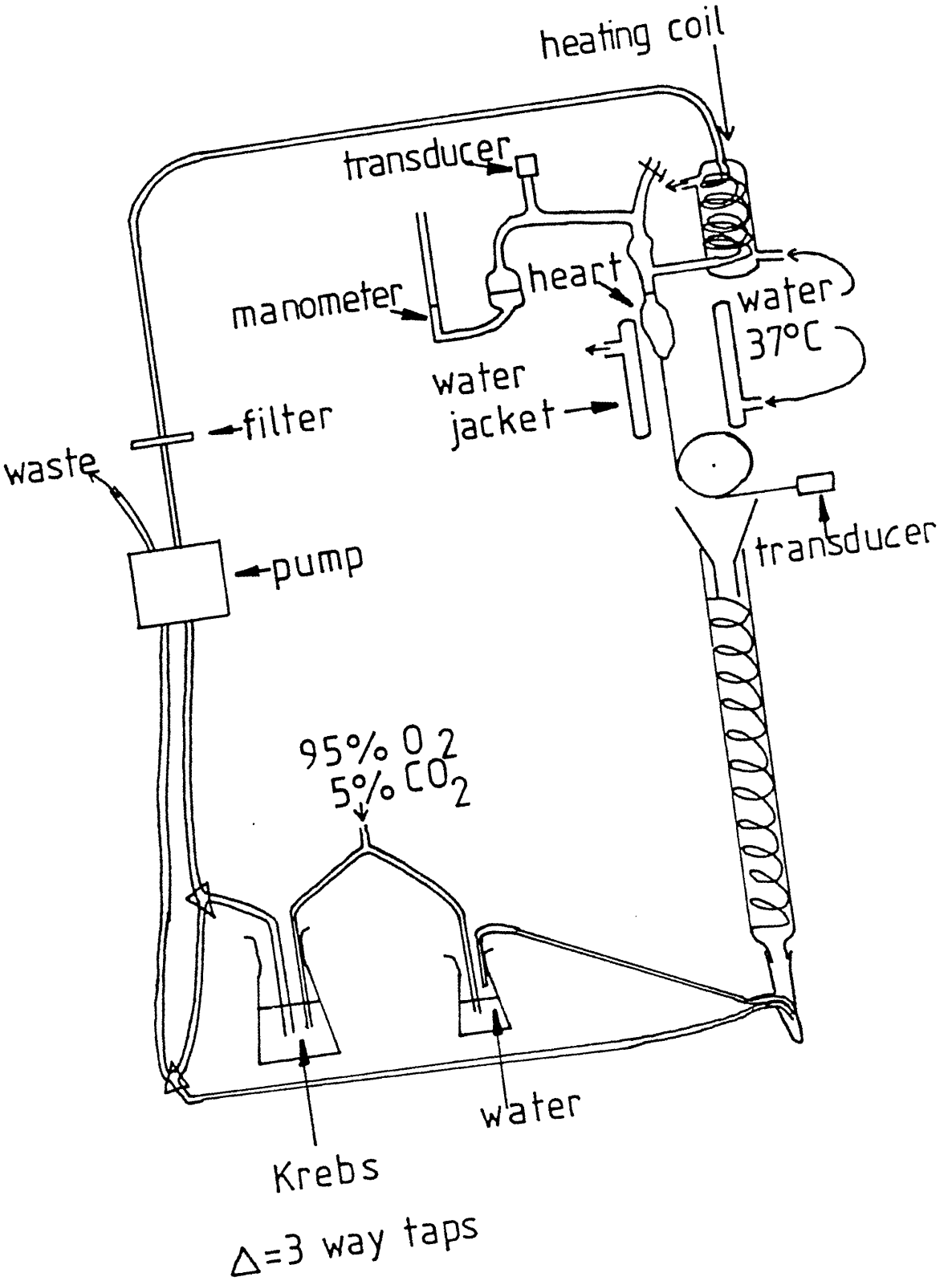


FIG 4. HEART PERFUSION SYSTEM

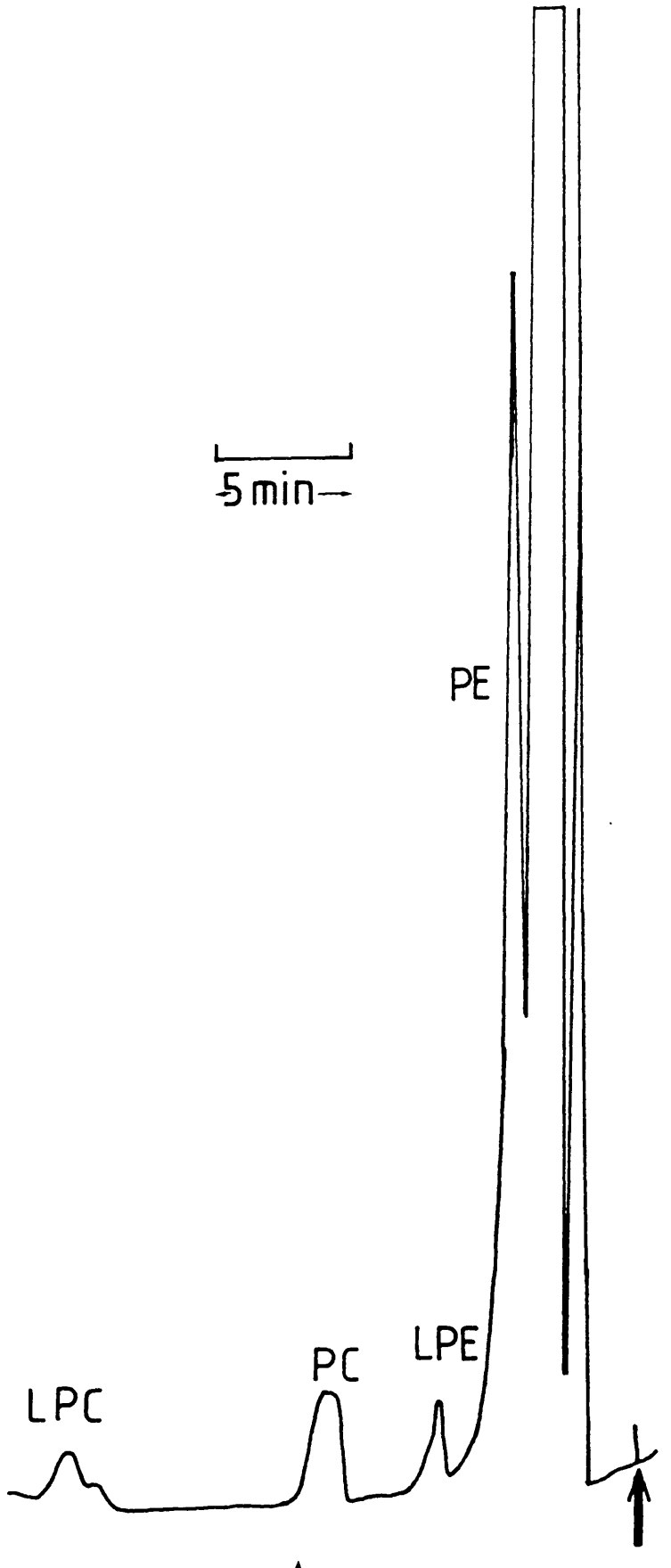


FIG 5. HPLC STANDARDS. ↑ INJECTION OF SAMPLE. MIXTURE OF PE, LPE, PC, AND LPC IN 20µl OF CHLOROFORM/METHANOL (2/1).

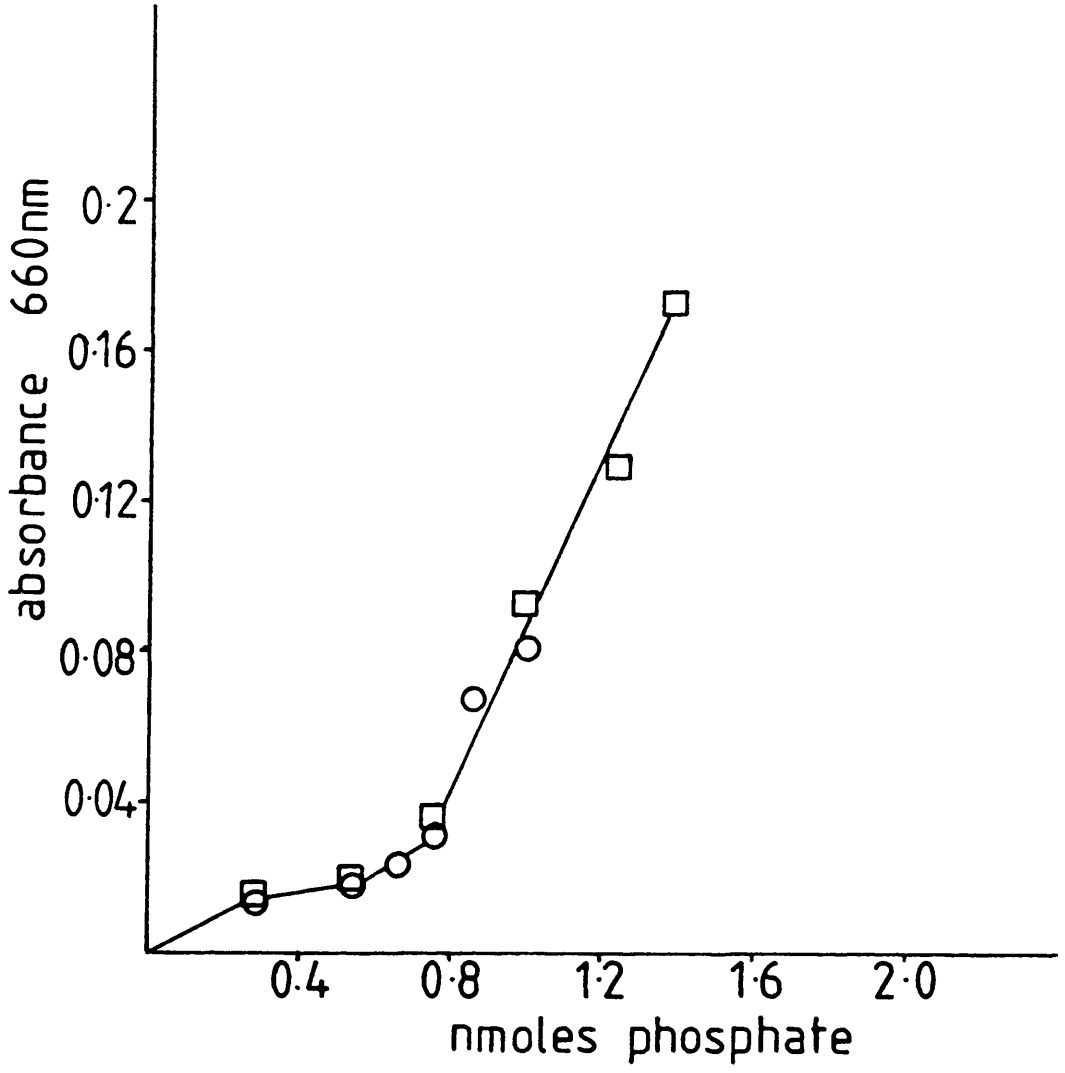


FIG 6. STANDARD PHOSPHATE CURVE FOR 1NMOL PHOSPHATE.

□ INORGANIC PHOSPHATE. FOR ≥ 0.5 NMOL r = 0.9991

○ ORGANIC PHOSPHATE. FOR ≥ 0.5 NMOL r = 0.9820

EACH POINT MEAN OF 2 SEPARATE ASSAYS.

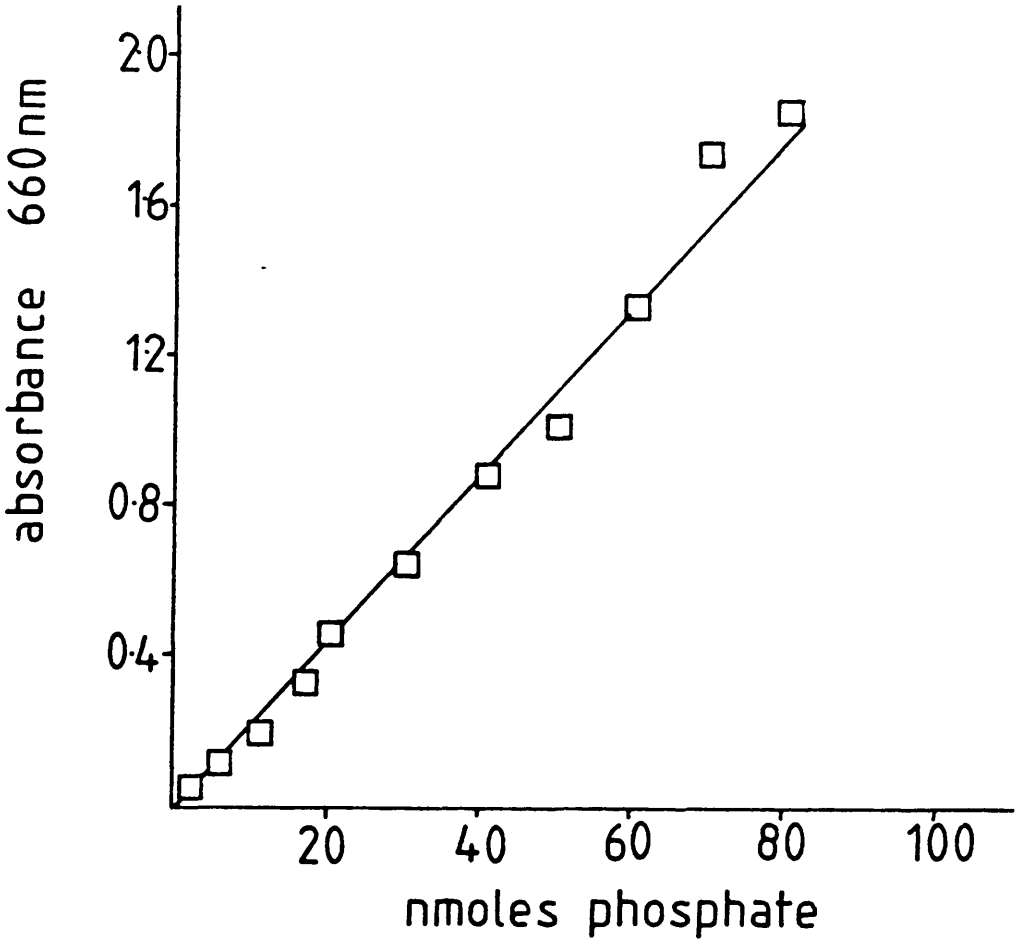


FIG 7. STANDARD CURVE FOR ORGANIC PHOSPHATE ≥ 1NMOL. r = 0.9963
EACH POINT MEAN OF DUPLICATES.

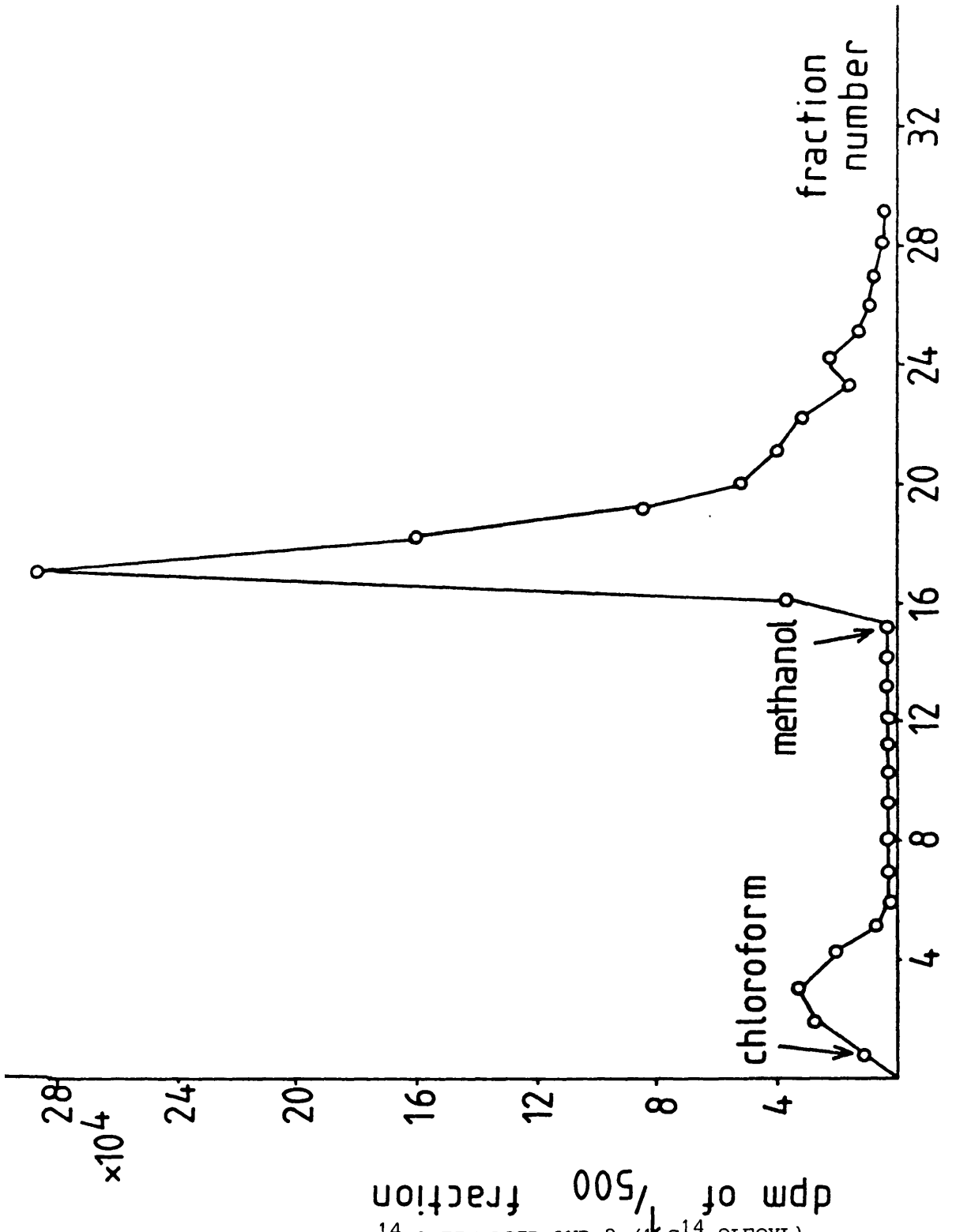


FIG 8. ELUTION OF C^{14}-OLEIC ACID AND 2-(1-C^{14} OLEOYL) PHOSPHATIDYLETHANOLAMINE FROM SILICIC ACID COLUMN.

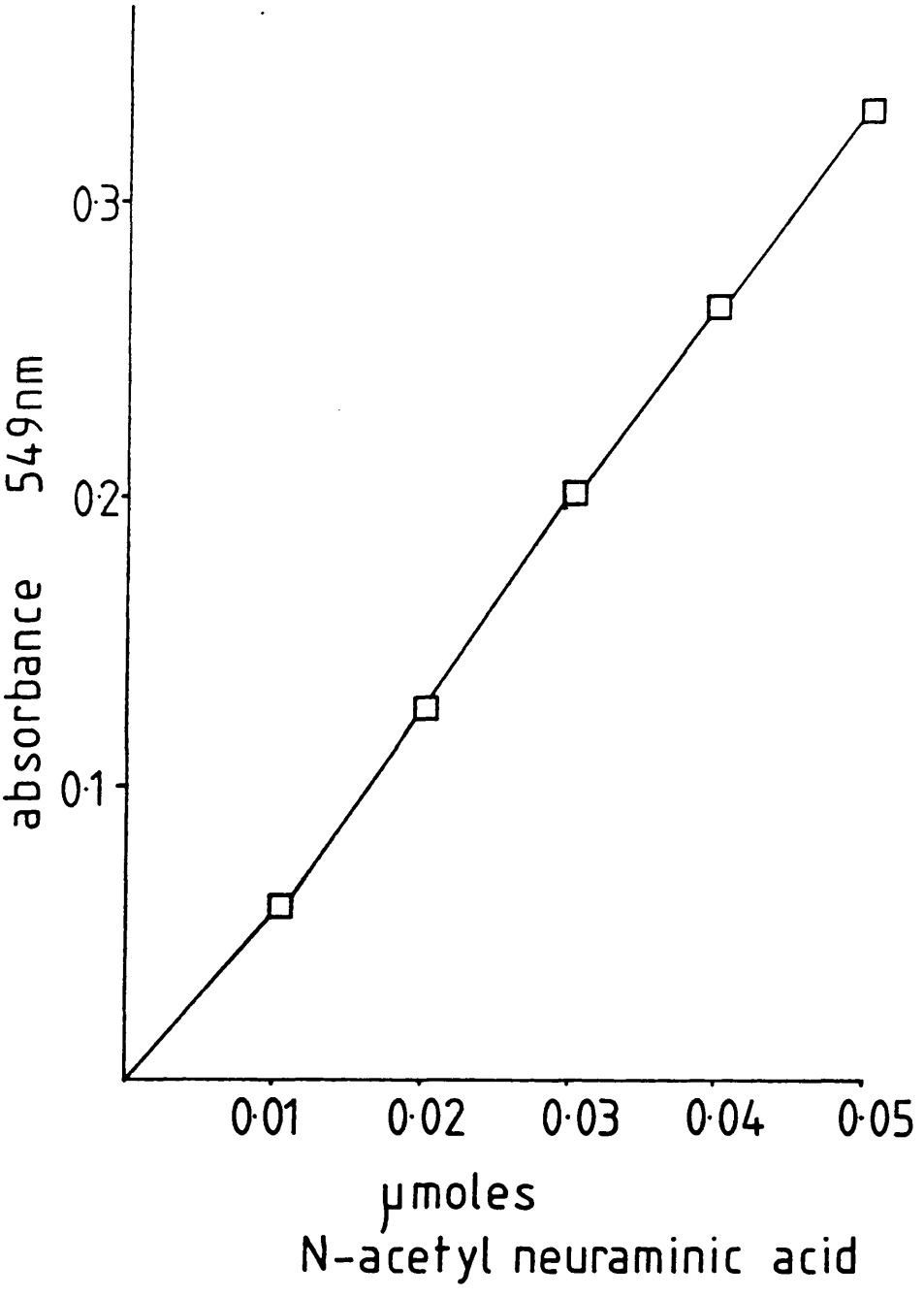
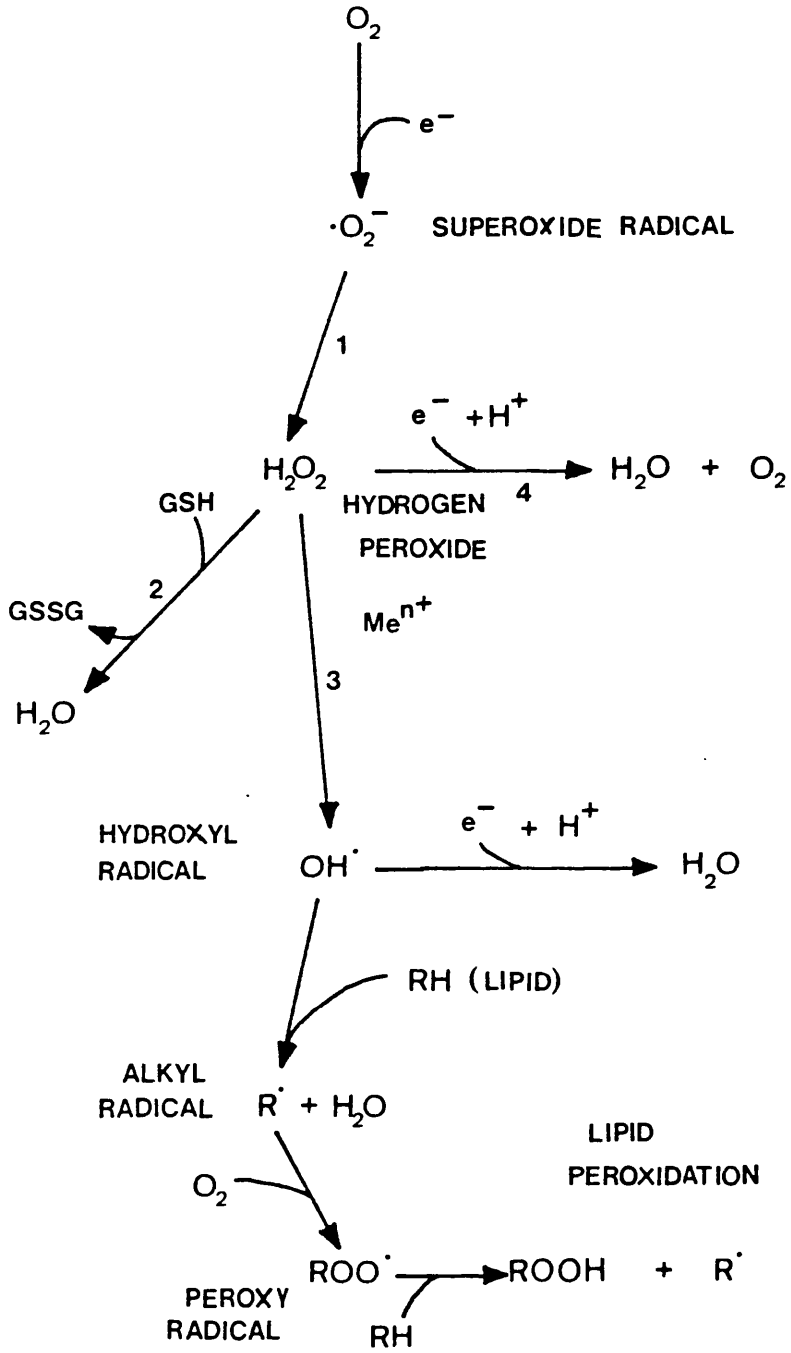


FIG 9. STANDARD CURVE FOR SIALIC ACID ASSAY.
r = 0.9996.



1 - superoxide dismutase 2 - glutathione peroxidase

3 - Fenton reaction 4 - catalase

FIG 10. PATHWAYS OF FREE RADICAL PRODUCTION AND SCAVENGING

RESULTS

7 MEASUREMENT OF PHOSPHOLIPIDS AND LYSOPHOSPHOLIPIDS IN THE ISOLATED RAT HEART

7.1 Phospholipid composition of the isolated rat heart after 10 minutes coronary artery ligation

Initially it was decided to measure the concentrations of lysophospholipids after a fixed period of ischaemia (10 minutes), to look at any changes in both the ischaemic and non-ischaemic regions.

Phospholipid composition was determined in the ischaemic and non-ischaemic areas of hearts subjected to 10 minutes coronary artery ligation or sham ligation for 10 minutes.

No difference was seen in composition between the ischaemic and non-ischaemic areas in each heart. However changes were seen between control and coronary artery ligated hearts (FIG 11). The means of the values for the ischaemic and non-ischaemic areas were obtained for comparison of the phospholipid compositions under ischaemic and control conditions (FIG 12). There was no significant change in PE or PC concentrations after 10 minutes coronary artery ligation in comparison to sham ligated controls (FIG 12).

In contrast, LPE and LPC both showed a significant increase in concentration following 10 minutes coronary

artery ligation, from 1.54 +/- 0.22 to 4.70 +/- 0.40 nmol/mg protein and 1.25 +/- 0.29 to 5.74 +/- 0.63 nmol/mg protein respectively. Ten minutes coronary artery ligation therefore produces an increase in lysophospholipid concentration in the isolated rat heart although this increase was not confined to the ischaemic area.

7.2 Time course of changes in phospholipid composition during ischaemia in the isolated rat heart

Although increases in LPC and LPE concentrations have been seen in a number of preparations during ischaemia and these have been implicated in the development of arrhythmias (see section 1.5 (3)) it is possible that this increase is produced either as a consequence of, or in parallel to, arrhythmias as opposed to being a causative factor in their production. Thus a time course of phospholipid concentration following coronary artery ligation was carried out to see if changes occurred before the onset of arrhythmias.

Phospholipid composition was determined in ischaemic tissue after periods of coronary artery ligation from 0 to 30 minutes. Sham ligated controls were also carried out.

No change was seen in PE or PC concentrations during the 30 minutes after coronary artery ligation (FIG 13) although there was a trend towards a slow oscillation of

the concentrations. Ten minutes ligation did produce a significant increase in PE concentration in comparison to time 0 but PE concentrations at other times were not significantly different from time 0. At 15 minutes control PE and PC concentrations were 25.69 +/- 2.0 and 24.49 +/- 1.45 nmol/mg protein respectively whilst at 30 minutes PE and PC concentration were 31.2 +/- 2.7 and 25.95 +/- 2.33 nmol/mg protein respectively.

There appeared to be an increase in LPE concentration at approximately 20 minutes post ligation but this was not statistically significant (FIG 14). The controls showed no difference from the time 0 LPE concentration , being 2.03 +/- 0.27, 1.65 +/- 0.144 and 2.09 +/- 0.3 nmol/mg protein at time 0, 15 and 30 minutes respectively.

LPC concentration had increased significantly by 5 minutes post ligation and remained elevated until 25 minutes post ligation, when the concentration fell to control levels (1.65 +/- 0.31 as opposed to 1.51 +/- 0.14 nmol/mg protein at time 0) (FIG 14). At 30 minutes however the LPC concentration had again increased to a value significantly greater than time 0. In contrast control hearts showed no difference from time 0 in LPC concentration, being 1.99 +/- 0.21 and 2.09 +/- 0.52 nmol/mg protein at 15 and 30 minutes respectively.

In this model arrhythmias were not precisely quantified but were seen as changes in the developed

tension and perfusion pressure traces and they typically commenced at 11 to 12 minutes post ligation, thus there was a significant increase in LPC concentration before the onset of arrhythmias. Arrhythmias ceased at 23 to 25 minutes post ligation and this coincided with the decrease in LPC concentrations to control levels at 25 minutes. The further increase in LPC at 30 minutes did not relate to the production of more arrhythmias at this time.

If the values for LPE and LPC are summed (FIG 15) there can be seen to be a significant increase in lysophospholipid concentration with time after coronary artery ligation until 25 minutes when the concentration approaches that of controls.

7.3 Effect of perfusate ionic changes on phospholipid composition in the isolated rat heart

The ionic composition of the perfusate affects the severity and time of onset of arrhythmias produced by coronary artery ligation and therefore it was decided to see if this relates to any changes in phospholipid concentration.

The effect of changes in the perfusate K^+ and Ca^{2+} concentrations on phospholipid composition in control hearts and hearts subjected to 20 minutes coronary artery ligation was determined (as 20 minutes was shown in the time course study to produce maximal changes in LPC and

LPE) .

No change was seen in PC or PE concentration in control hearts with any of the buffers in comparison to the standard buffer (FIG 16). Similarly 20 minutes coronary artery ligation did not produce any change in the PC or PE concentration with any of the four buffers (FIG 17).

LPE concentration did not change in controls with any of the buffers and no change was seen after 20 minutes coronary artery ligation (FIG 18).

Thus in control and coronary artery ligated hearts there were no significant differences in PE, PC, or LPE concentrations when the ionic composition of the Krebs-Henseleit buffer was changed by increasing the calcium and/or decreasing the potassium concentrations.

In contrast, in non-ligated control hearts changes in perfusate ionic composition caused changes in the concentration of LPC (FIG 19). An increase in Ca^{2+} concentration from 1.25mM to 2.5mM caused, at a constant potassium concentration of either 5.9mM or 2.5mM, an increase in the concentration of LPC. Although a decrease in K^{+} concentration at constant calcium concentration appeared to produce an increase in LPC concentration this was not significant (FIG 19).These increases in LPC in control hearts were similar to those seen after 20 minutes coronary artery ligation in the standard Krebs-Henseleit buffer (FIG 20) implying that a maximal LPC

concentration might have been reached by either increasing perfusate Ca^{2+} concentration or by coronary artery ligation. Twenty minutes coronary artery ligation produced slight increases in LPC concentration with all the buffers. These were not significant , except with the standard Krebs-Henseleit (FIG 20).

Increasing Ca^{2+} concentration thus produces an increase in total lysophospholipid concentrations (FIG 21) in control hearts. The concentration of LPC plus LPE after 20 minutes coronary artery ligation was not significantly different between the varying buffers. when e.w. their controls

Therefore changes in Ca^{2+} and K^{+} concentration of the perfusing buffer which make the isolated rat (Woodward, Hassan and Mohamed 1983) or rabbit heart (Karki 1958) more susceptible to ischaemically induced arrhythmias lead to increased basal levels of lysophospholipids, in particular LPC, although they do not change tne concentration seen after 20 minutes coronary artery ligation. These changes in ionic composition do not produce any further increase in lysophospholipids above that produced by coronary artery ligation.

7 DISCUSSION

MEASUREMENTS OF PHOSPHOLIPIDS AND LYSOPHOSPHOLIPIDS IN THE ISOLATED RAT HEART

Phospholipid composition of the isolated rat heart after 10 minutes coronary artery ligation

An increase in lysophospholipid concentration in the ischaemic area of the isolated rat heart was seen following coronary artery ligation for 10 minutes. This increase was also seen in the non-ischaemic area (FIG 11). This result was surprising as no change would be expected in the non-ischaemic area. However such a change was also seen by Shaikh and Downar (1981) in the *in vivo* pig heart following 40 minutes ischaemia, with an increase from 40.3 +/- 1.3 to 51.0 +/- 2.6 nmol/ g wet weight in the non-ischaemic area compared to 55.2 +/- 2.6 nmol/ g wet weight in the ischaemic area. Increases in LPC concentration in the non-ischaemic area may be due to collateral flow, washout from the ischaemic to the non-ischaemic area occuring. Collateral flow in the rat has been estimated to be 18% (Kannengiesser, Lubbe and Opie 1975) although other workers have concluded it is much smaller (Schaper 1984). Collateral flow in the pig is also thought to be small (1 to 10%) (Bloor and White 1982) therefore collateral flow is unlikely to account for LPC concentrations increasing in the non-ischaemic

area to levels approaching those in the ischaemic area. However, washout of lactate from tne ischaemic area has been demonstrated in a similar preparation of the isolated rat heart (Daugherty, Frayn, Redfern and Woodward 1986) and as a recirculating system was used in this study it is possible that washout of LPC to the perfusate followed by recirculation to the non-ischaemic area could occur and contribute to the raised LPC concentrations in this area.

In the remaining work concentrations of lysophospholipids following coronary artery ligation were studied in the ischaemic area in comparison to sham ligated controls.

SUMMARY: Coronary artery ligation produces increases in lysophospholipids in both tne ischaemic and non-ischaemic regions of the isolated rat heart, although the increase in the latter may be an artefact of using a recirculating perfusion system.

Time course of changes in phospholipid composition during ischaemia in the isolated rat heart

Over a time course of 30 minutes following coronary artery ligation there was a significant increase in LPC concentration. The control concentration of LPC, prior to ligation, was 1.51 +/- 0.14 nmol/mg protein, similar to that seen in the dog (LPC = 1.0 +/- 0.21 nmol/mg protein, Man and Slater et.al. 1983) and the cat myocardium (LPC =

2.8 +/- 0.23 nmol/mg protein, Corr and Snyder et.al. 1982) but greater than that in the pig (LPC = 40.3 +/- 1.3 nmol/g wet weight, i.e. approx. 0.2nmol/mg protein, Shaikh and Downar 1981). This may reflect methodological differences or alternatively be due to species difference.

The control concentration of LPE found in the isolated rat heart (2.03 +/- 0.27 nmol/mg protein) was similar to that reported in the cat (2.1 +/- 0.12 nmol/mg protein, Corr and Snyder et.al.1982) and the dog myocardium (0.51 +/- 0.07 nmol/mg protein, Man and Slater et.al. 1983).

LPC concentration was increased within 5 minutes following coronary artery ligation and thus as the concentration had increased before the onset of arrhythmias, which commenced at 11 to 12 minutes following ligation, it is unlikely that increased lysophospholipid concentrations are a result of the arrhythmias. This is supported by the failure of electrically induced ventricular fibrillation in anaesthetized cats to increase lysophospholipid concentrations (Corr, Sharma and Sobel 1983). As lysophospholipid concentrations increase prior to the development of arrhythmias it does not exclude the possibility that they may have a causative role in their development. LPC concentrations continued to increase until 25 minutes following coronary artery ligation, at which time the concentration was not significantly

different from time 0. This coincides with the cessation of arrhythmias and may represent the loss of cellular integrity and washout of lipids from the ischaemic region. A similar decrease in the LPC concentration of rat heart mitochondrial phospholipids has been reported following 25 minutes global ischaemia (Victor, van der Merwe, Benade, LaCock and Lochner 1985). In this study an increase in LPC occurred from 0 to 15 minutes which was followed by a fall below control values at 25 minutes. The same pattern was seen by these workers in the concentration of LPE.

Following the decrease in LPC at 25 minutes there was a further increase at 30 minutes. As this was little different from the concentration at 20 minutes it may represent a maximal concentration similar to that seen in the pig myocardium (Shaikh and Downar 1981) following 8 minutes coronary artery ligation with the establishment of an equilibrium between production and metabolism. This further increase at 30 minutes post ligation did not correlate with any further production of arrhythmias. This may be caused by the lack of excitability of the cells following prolonged ischaemia and thus any effects of LPC on ion movement would be of no importance.

In contrast to LPC, LPE showed little change in concentration over the time course of 30 minutes although there was a trend to increased LPE concentrations from 15 to 20 minutes prior to a decrease to control levels by 25

minutes ligation. With the exception of the controls there was a large degree of variability between different hearts which led to large standard errors. This probably reflects the inhomogeneities in response between different hearts to the same ischaemic insult and may also reflect differences in collateral flow which could allow both washout of lysophospholipids and cause variation in the size of tne ischaemic area. It is unlikely to be due to variations in basal levels between different hearts or methodological errors as control hearts showed markedly less variation.

Control LPC and LPE values from 0 to 30 minutes showed no significant change in comparison to time 0 therefore the increase in lysophospholipids seen is not a progressive increase in phospholipid breakdown due to the perfusion system used.

The sum of LPC and LPE increased following coronary artery ligation (FIG 15). By 15 minutes post ligation a 50% increase had occurred which is of the same order as that seen in the cat myocardium where an increase of 53% occurred following 10 minutes coronary artery ligation (Corr and Snyder et.al. 1982), and in the pig myocardium where there was an increase of 46% following 12 minutes coronary artery ligation (Shaikh and Downar 1981).

No significant changes were seen in PC concentration following ligation. This is despite a significant increase in LPC which is probably formed by deacylation

of PC. The lack of an observed decrease in PC could be due to the high concentrations of the phospholipid in comparison to its lyso- derivative, as only a small amount of PC degradation could lead to a significant increase in LPC. There was likewise no decrease in PE concentration over the time course studied. These findings are in agreement with those of Shaikh and Downar (1981), Corr and Snyder et.al.(1982), Chien et.al. (1984), and Steenbergen and Jennings (1984), all of whom found no change in total phospholipid or PE and PC concentrations following ischaemia lasting up to 1 hour. Longer periods of ischaemia have resulted in decreases in phospholipids in the latter two reports and these changes have been suggested to be due to the release of lysosomal enzymes (Wildenthal 1978) or tissue oedema as concentrations are often quoted per gram wet weight (Man and Slater et.al. 1983, Steenbergen and Jennings 1984).

There did appear to be an oscillation in PC and PE concentration over the 30 minute time course which may represent phospholipid turnover in the cell membrane. This type of oscillatory trend has been shown with other compounds previously thought to be constant in the cell such as adenine nucleotides (Mowbray, Bates and Perrett 1981).

SUMMARY: Lysophospholipid concentrations increase significantly before the onset of arrhythmias indicating that they could have a role to play in their development

and it is unlikely that their levels increase as a consequence of the arrhythmias. The concentration of lysophospholipids appeared to reach a maximum, possibly due to the establishment of an equilibrium between their production and metabolism.

Effect of perfusate ionic changes on phospholipid composition in the isolated rat heart

Changes in the ionic composition of the perfusate caused changes in the lysophospholipid concentrations in sham ligated controls. Although no change in LPE concentration was seen between the various buffers a significant increase in LPC concentration was seen when the calcium concentration of the buffer was increased from 1.25mM to 2.5mM at a constant potassium concentration. Normal ionised blood calcium is approximately 1mM (Poole-Wilson, Harding, Bourdillon and Tones 1984) and in isolated heart myocytes this extracellular calcium concentration has been shown to produce an intracellular calcium concentration of 0.2μM (Klein, Snowdowne and Borle 1982). Increases in external calcium concentration have been shown to cause an increase in intracellular free calcium in the sheep papilliary muscle (Sheu and Fozzard 1982), rabbit heart (Bridge and Bassingthwaite 1982), and ferret ventricular muscle (Allen, Eisner, and Orchard 1984) and in isolated

smooth muscle cells where an increase in external calcium concentration from 1.25mM to 2.5mM produced an increase in cytoplasmic calcium concentration from approximately 0.125μM to 0.15μM (Williams, Fogarty, Tsien and Fay 1985). Increased intracellular calcium may cause increased phospholipid breakdown as membrane bound phospholipases are calcium dependent (review van den Bosch 1980), alternatively there may be a decreased reacylation of LPC as calcium has been shown to inhibit some of the enzymes involved with this process (Arthur and Choy 1986). The increases in cytosolic free calcium seen on raising the external calcium concentration in isolated ventricular cells were proportional to the change in external calcium (Snowdowne, Ertel and Borle 1985) as were changes in total cell calcium (Cheung, Leaf and Bonventre 1984) however in the latter case free calcium was not measured and there is no good correlation between free and total intracellular calcium content (review Cheung, Bonventre, Malis and Leaf 1986).

The increase in cellular free calcium is not likely to be large enough to produce effects on phospholipid metabolism if the calcium concentration is assumed to be homogenous in the cell (although local concentrations near the sarcolemma may be initially greater) as the Km for the enzymes are in the millimolar range, substantially greater than the internal free calcium concentration. Subcellular regions of locally elevated intracellular calcium rather than homogenously elevated

calcium transients have been demonstrated in isolated rat heart cells (Berlin, Cannell, Goldman, Lederer, Marban and Wier 1985) and in smooth muscle cells (Williams et.al. 1985) using digital imaging of Fura-2 fluorescence and thus an elevated local concentration of calcium is possible.

LPC concentrations also appeared to increase when the calcium concentration was kept constant but the potassium concentration of the perfusate was decreased from 5.9mM to 2.5mM although this was not statistically significant. A reduction in extracellular potassium is likely to cause some inhibition of the Na^+ / K^+ ATPase, a rise in intracellular sodium and therefore of calcium by Na^+ / Ca^{2+} exchange as shown by Hoerter, Miceli, Renlund, Jacobus, Gerstenblith and Lakatta (1986) in the Langendorff perfused rat heart.

Thus increases in external calcium concentration and decreases in external potassium concentration may be acting by the same mechanism to increase intracellular free calcium.

Following coronary artery ligation for 20 minutes with increased Ca^{2+} or decreased K^+ concentrations there were slight increases in LPC which were not statistically significant, possibly due to the large numbers needed to achieve significance because of the variability in LPC concentrations seen.

When the concentrations of LPC and LPE were summed

there can be seen to be an increase in lysophospholipids in control hearts when the perfusate calcium concentration is increased or the potassium concentration is decreased (FIG 21). Thus changes in the perfusate ionic composition that cause an increase in the severity and incidence of ischaemically induced arrhythmias (Woodward et.al. 1983) or electrically induced arrhythmias (Karki 1958) cause increases in lysophospholipid concentration in the isolated rat heart independent of coronary artery ligation.

With both the time course of lysophospholipid concentration changes during ischaemia and the perfusate ionic changes, the changes in LPC were more marked than those in LPE. This could reflect either;

a) increased specificity of the deacylating enzyme for PC
b) increased specificity of the reacylating enzymes for LPE
c) functional compartmentation of PC and PE

a) PE has been shown to be a better substrate for phospholipase A_2 when added exogenously (Weglicki et.al. 1971, Wurl and Kunze 1985). However in cases where phospholipid concentration has been shown to decrease during ischaemia both PC and PE have been equally affected (Man and Slater et.al. 1983, Steenbergen and Jennings 1984, Finkelstein, Gilfor and Farber 1985). However these decreases were following long periods of

ischaemia when lysosomal lipases may be of importance. This would suggest that there is unlikely to be a selective degradation of PC, as opposed to PE, during ischaemia although in one report PC depletion was been shown to surpass that of PE in canine sarcoplasmic reticulum following 30 minutes ischaemia (Yanagishita, Katagiri, Mochizuki, Umezu, Kitsu, Geshi, Konno, Tanno, Akiyama, Sekita and Niitani 1986).

b) The deacylation-reacylation reactions for PC and PE are suggested to change in ischaemia (Shaikh and Downar 1985) with differences between PC and PE occuring. It is also known that plasmalogen catabolism, which would lead to the production of lysophospholipids, occurs by different pathways for PC and PE (Arthur et.al. 1986) with plasmenylcholine being catabolised by phospholipase A_2 and plasmenylethanolamine by plasmalogenase. This may result in differential breakdown and reacylation.

Although from this evidence it appears unlikely that PC breakdown would surpass that of PE functional compartmentalisation of PC with phospholipase A_2 may still exist. Evidence for this is difficult to interpret but it is known that the distribution of phospholipids within the lipid bilayer is not homogenous (Op den Kamp, Roelofsen and van Deenan 1985) and there is an asymmetrical distribution of phospholipids on the inner and outer bilayer (Rothman and Lenard 1977). Purified enzymes and enzymes supplied with exogenous substrates

may show different substrate specificity to enzymes acting on substrates in the same membranes.

SUMMARY: LPC concentrations are increased in control hearts by changes in perfusate ionic composition that increase the severity of ischaemically induced arrhythmias. This may be secondary to an increase in intracellular free calcium concentration which activates phospholipase A_2 and/or inhibits reacylation of LPC although evidence suggests that the increase in intracellular calcium would not be large enough if distributed homogenously. Greater changes in LPC than in LPE may reflect differences in metabolism of PC and PE and their lyso- derivatives or functional compartmentalisation.

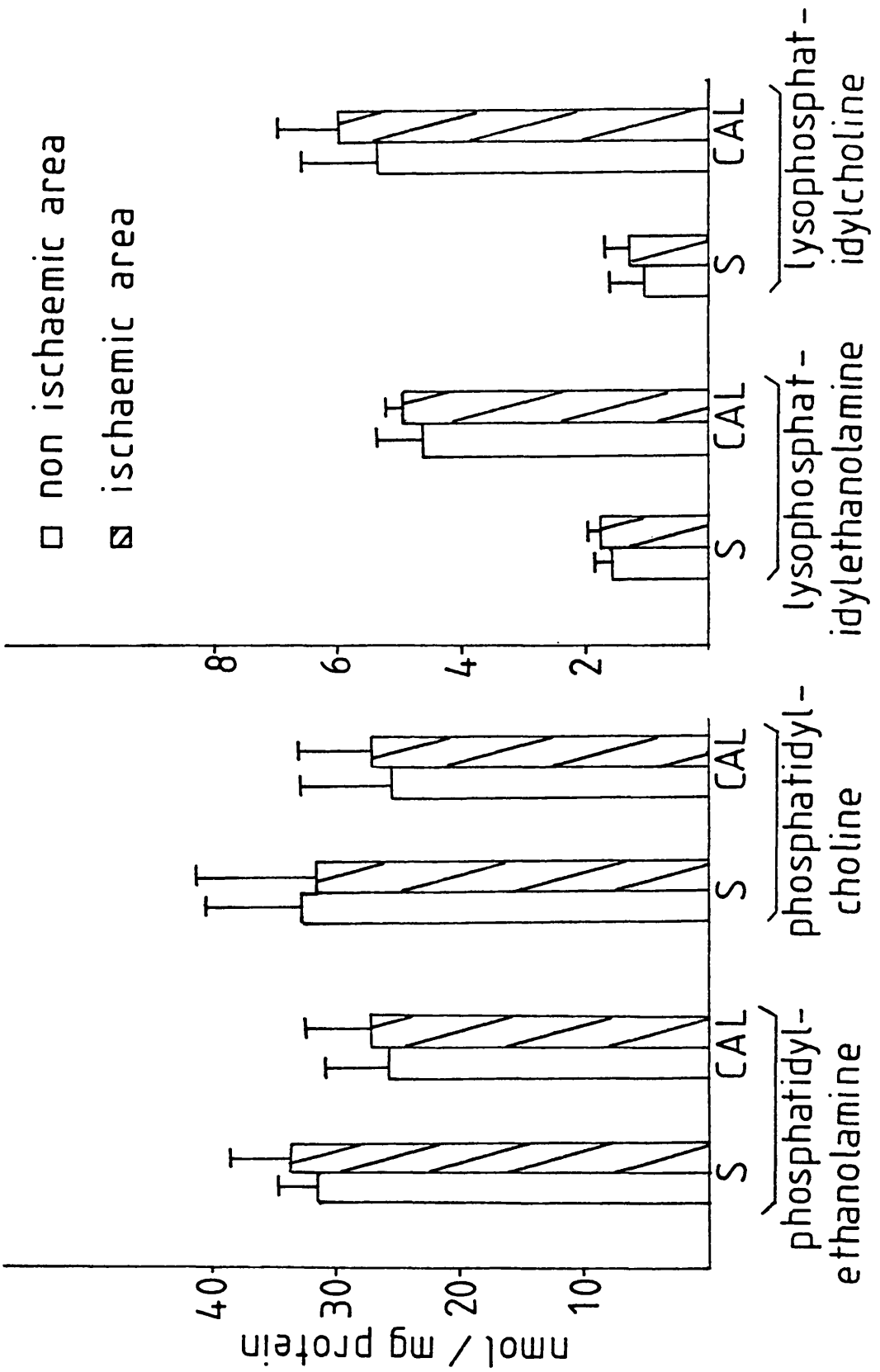


FIG 11. PHOSPHOLIPID CONCENTRATIONS IN THE ISOLATED RAT HEART AFTER 10 MINUTES CORONARY ARTERY LIGATION. MEAN +/- SEM. n=3-5. S = SHAM LIGATED. CAL = CORONARY ARTERY LIGATED.

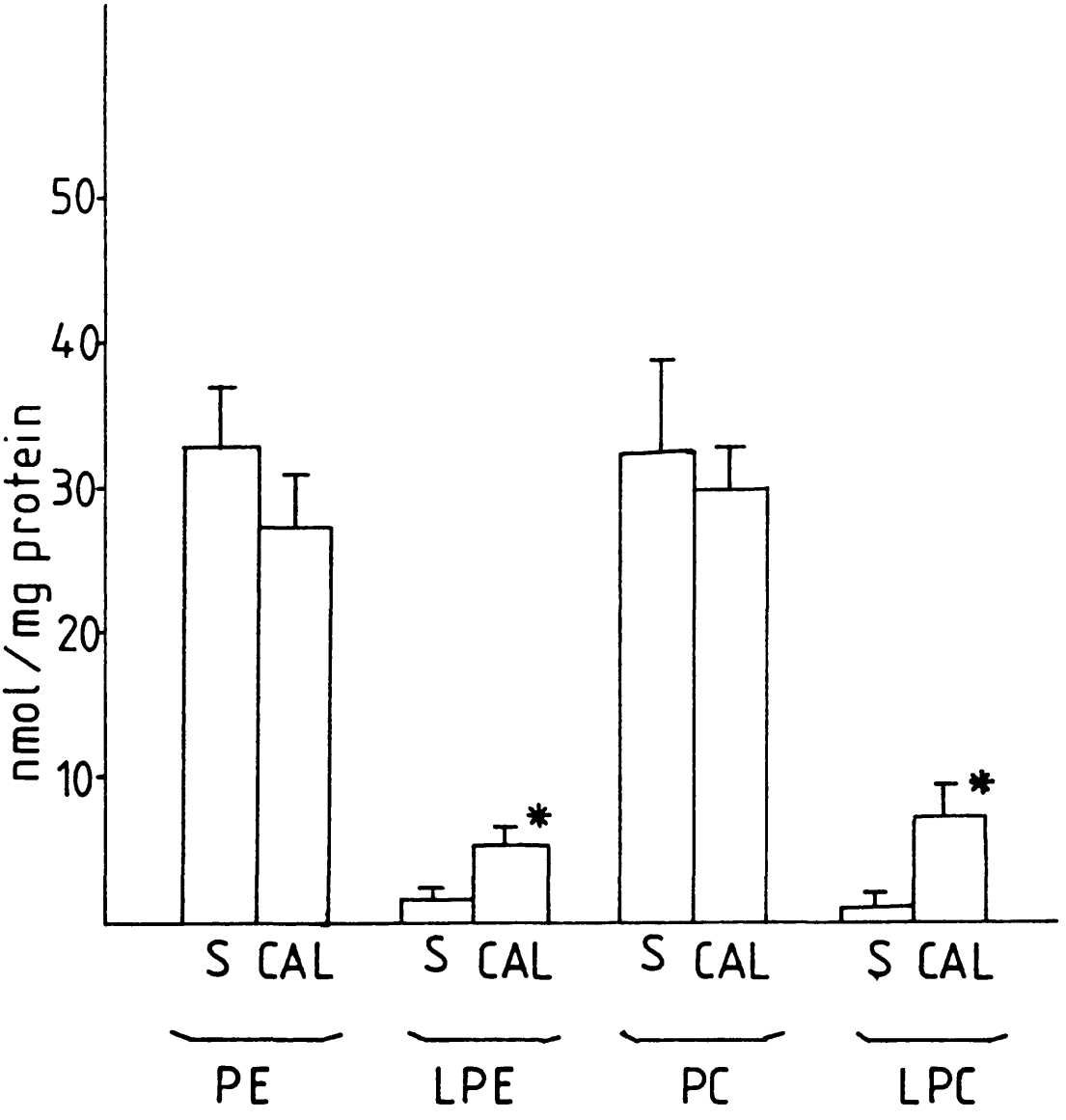


FIG 12. PHOSPHOLIPID CONCENTRATIONS IN THE ISOLATED RAT HEART FOLLOWING 10 MINUTES CORONARY ARTERY LIGATION. MEAN +/- SEM. PE = PHOSPHATIDYLEHANOLAMINE LPE = LYSOPHOSPHATIDYLETHANOLAMINE PC = PHOSPHATIDYLCHOLINE LPC = LYSOPHOSPHATIDYLCHOLINE. n = 5. S = SHAM LIGATED. CAL = CORONARY ARTERY LIGATED.
* $p \leq 0.01$ vs SHAM LIGATED.

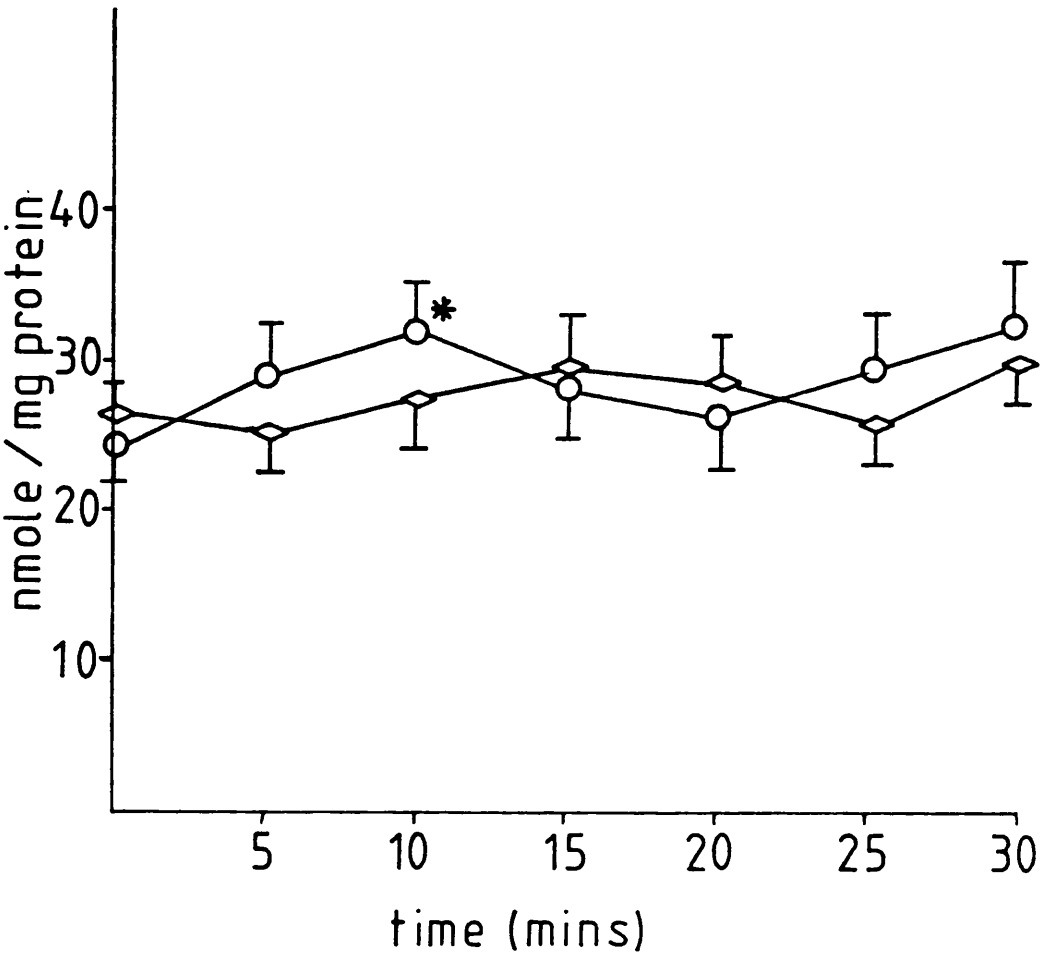


FIG 13. TIME COURSE OF PHOSPHOLIPID CONCENTRATION FOLLOWING CORONARY ARTERY LIGATION.

○ PE

◇ PC

MEAN +/- SEM. n = 12

* $p \leq 0.05$ vs TIME 0

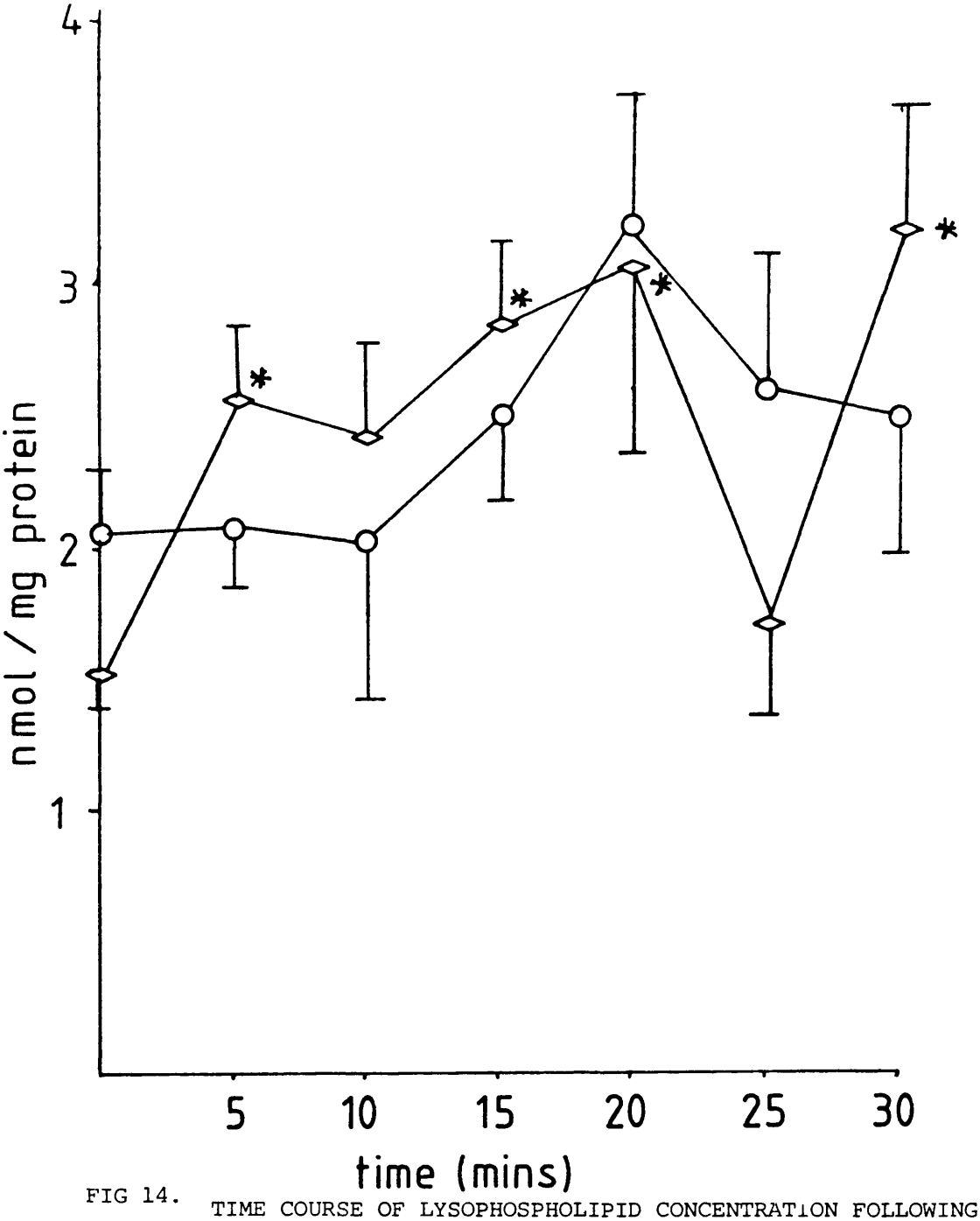


FIG 14. TIME COURSE OF LYSOPHOSPHOLIPID CONCENTRATION FOLLOWING CORONARY ARTERY LIGATION.

○ LPE

◇ LPC

MEAN +/- SEM. n = 12 * $p \leq 0.05$ vs TIME 0

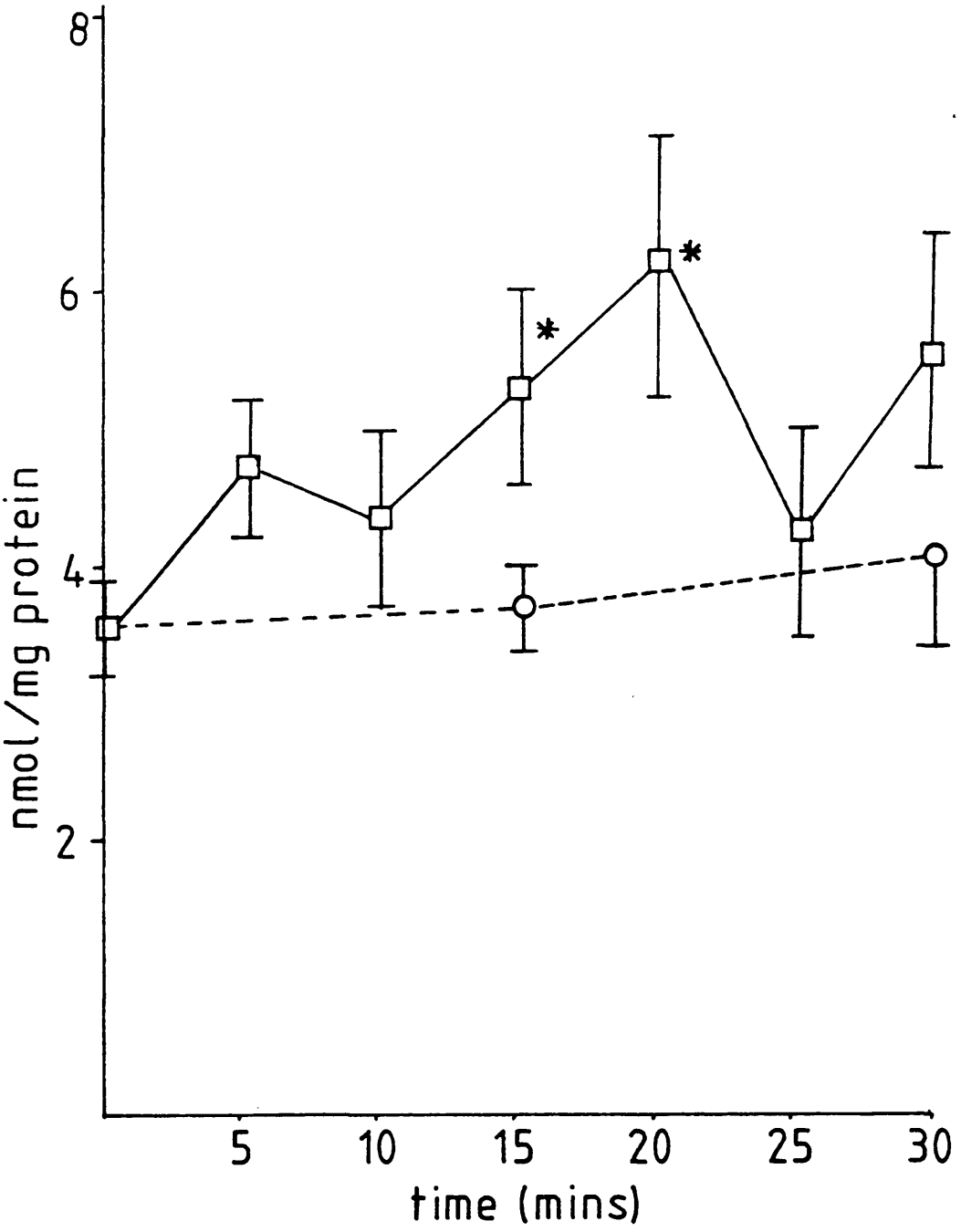


FIG 15. SUM OF LPE AND LPC CONCENTRATIONS WITH TIME FOLLOWING CORONARY ARTERY LIGATION.

□ LPE + LPC CORONARY ARTERY LIGATED.

○ LPE + LPC SHAM LIGATED CONTROLS.

MEAN +/- SEM. n = 12 * $p \leq 0.05$ vs TIME 0.

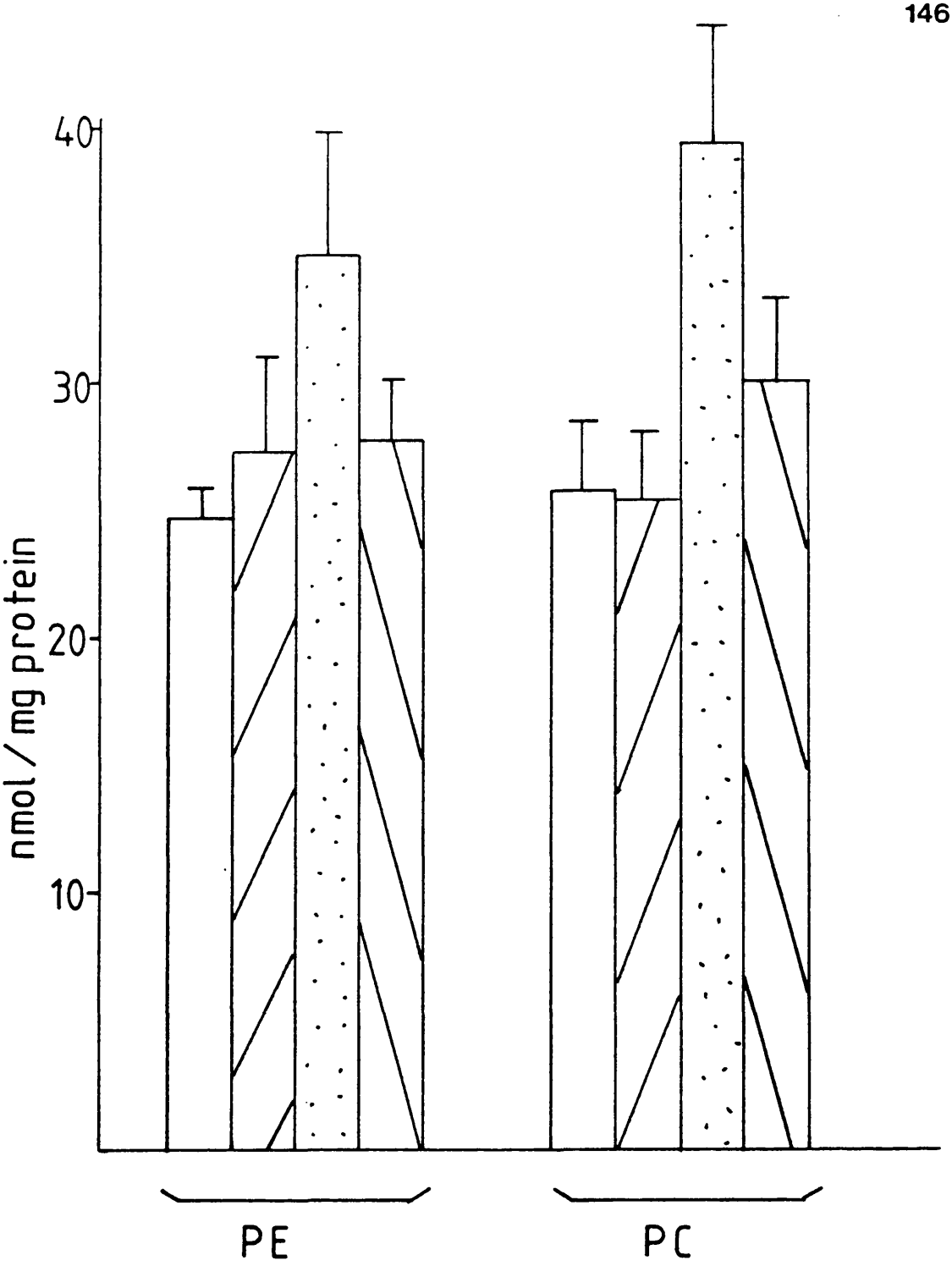


FIG 16. PHOSPHOLIPID CONCENTRATION IN SHAM LIGATED CONTROLS SUBJECTED TO DIFFERENT COMPOSITION BUFFERS. MEAN +/- SEM.

- 1.2mM Ca^{2+}, 5.9mM K^{+} n = 12
- 1.2mM Ca^{2+}, 2.5mM K^{+} n = 4
- 2.5mM Ca^{2+}, 5.9mM K^{+} n = 4
- 2.5mM Ca^{2+}, 2.5mM K^{+} n = 4

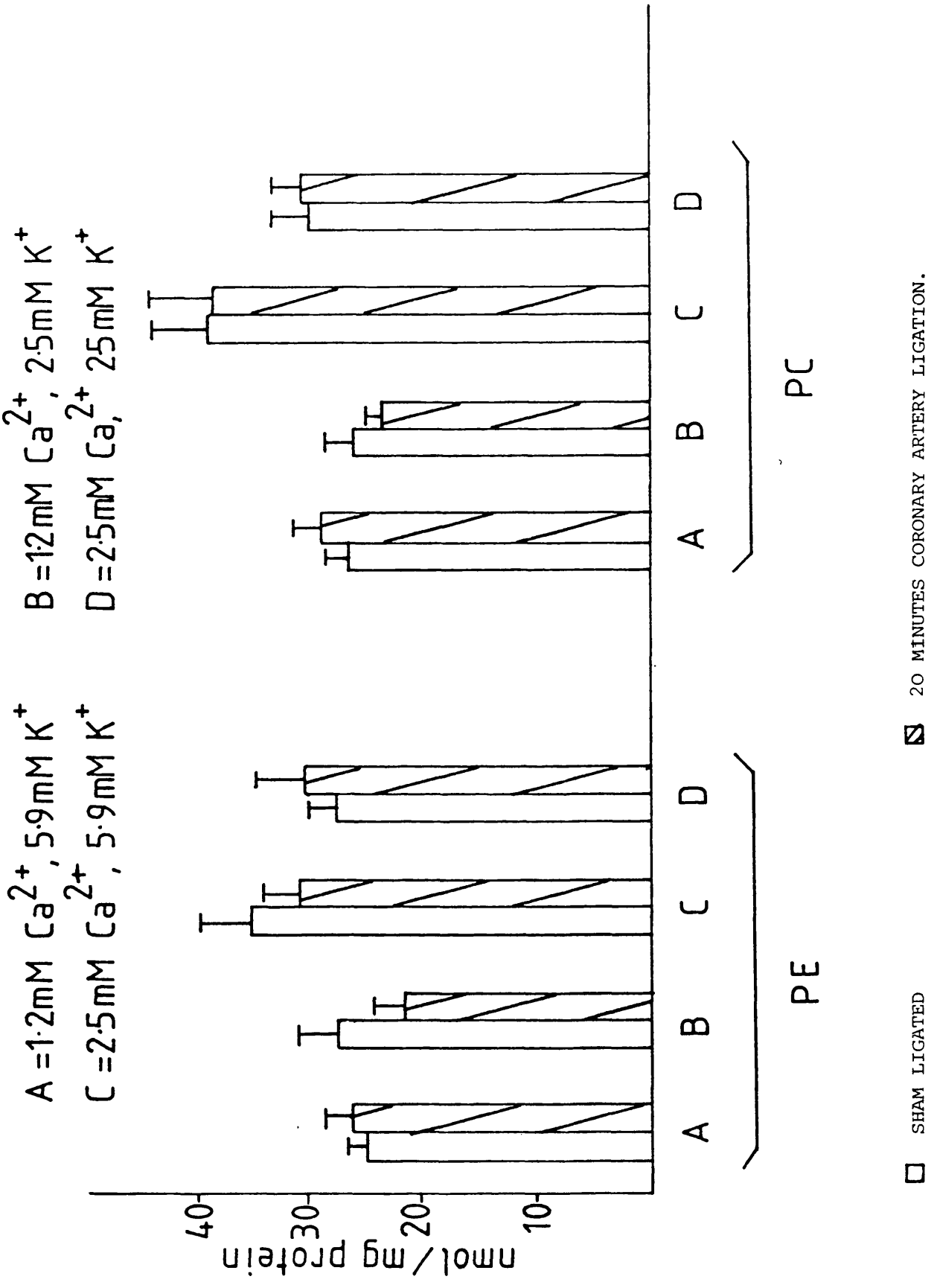


FIG 17. PHOSPHOLIPID CONCENTRATIONS FOLLOWING 20 MINS. CORONARY ARTERY LIGATION WITH DIFFERENT COMPOSITION BUFFERS. MEAN +/- SEM. A : n = 12, B-D n = 4.

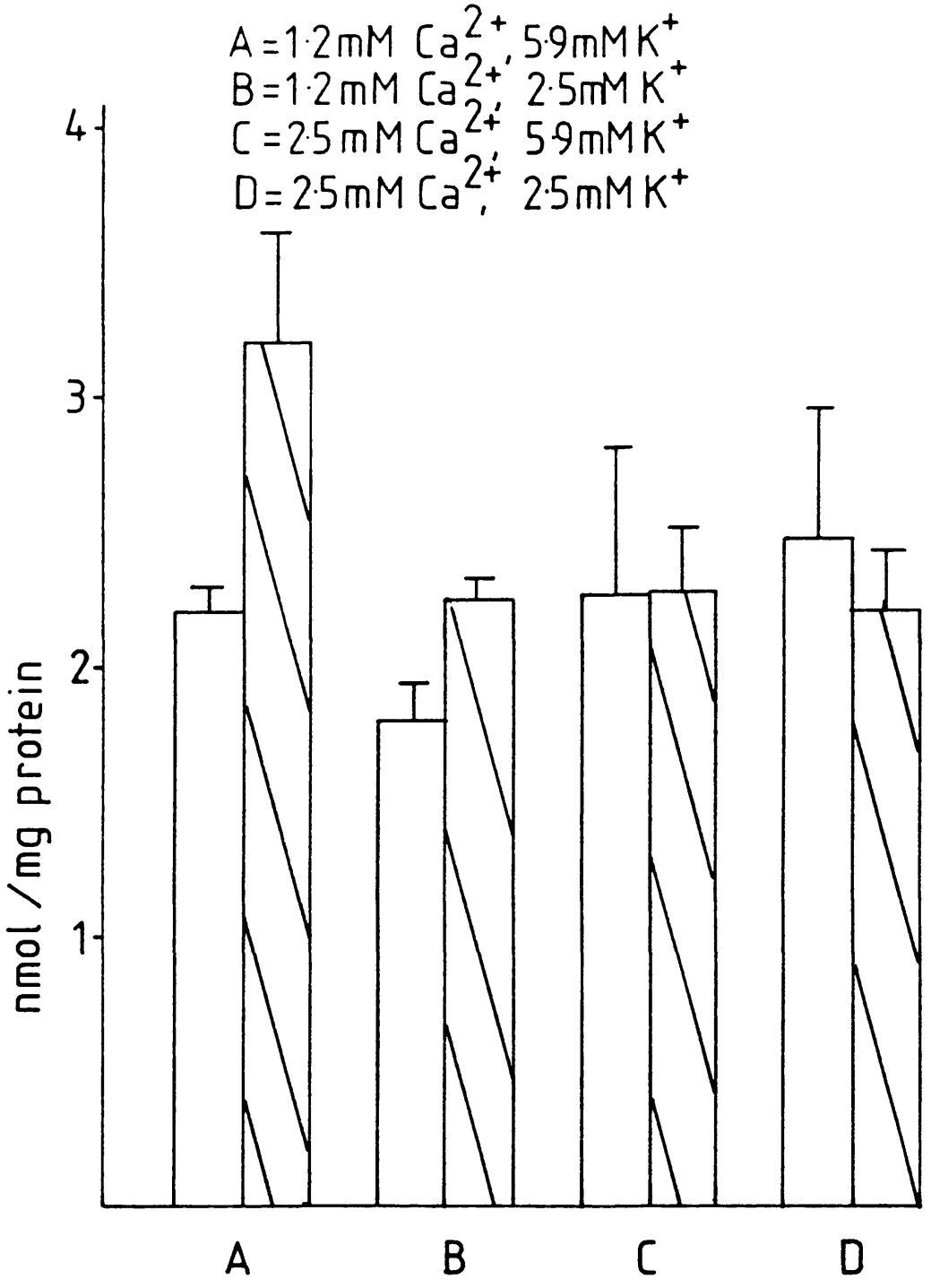


FIG 18. CHANGES IN LPE CONCENTRATION FOLLOWING 20 MINUTES CORONARY ARTERY LIGATION WITH DIFFERENT IONIC COMPOSITION BUFFERS.

MEAN +/- SEM. A n = 12. B-D n = 4

□ SHAM LIGATED. ▨ CORONARY ARTERY LIGATED.

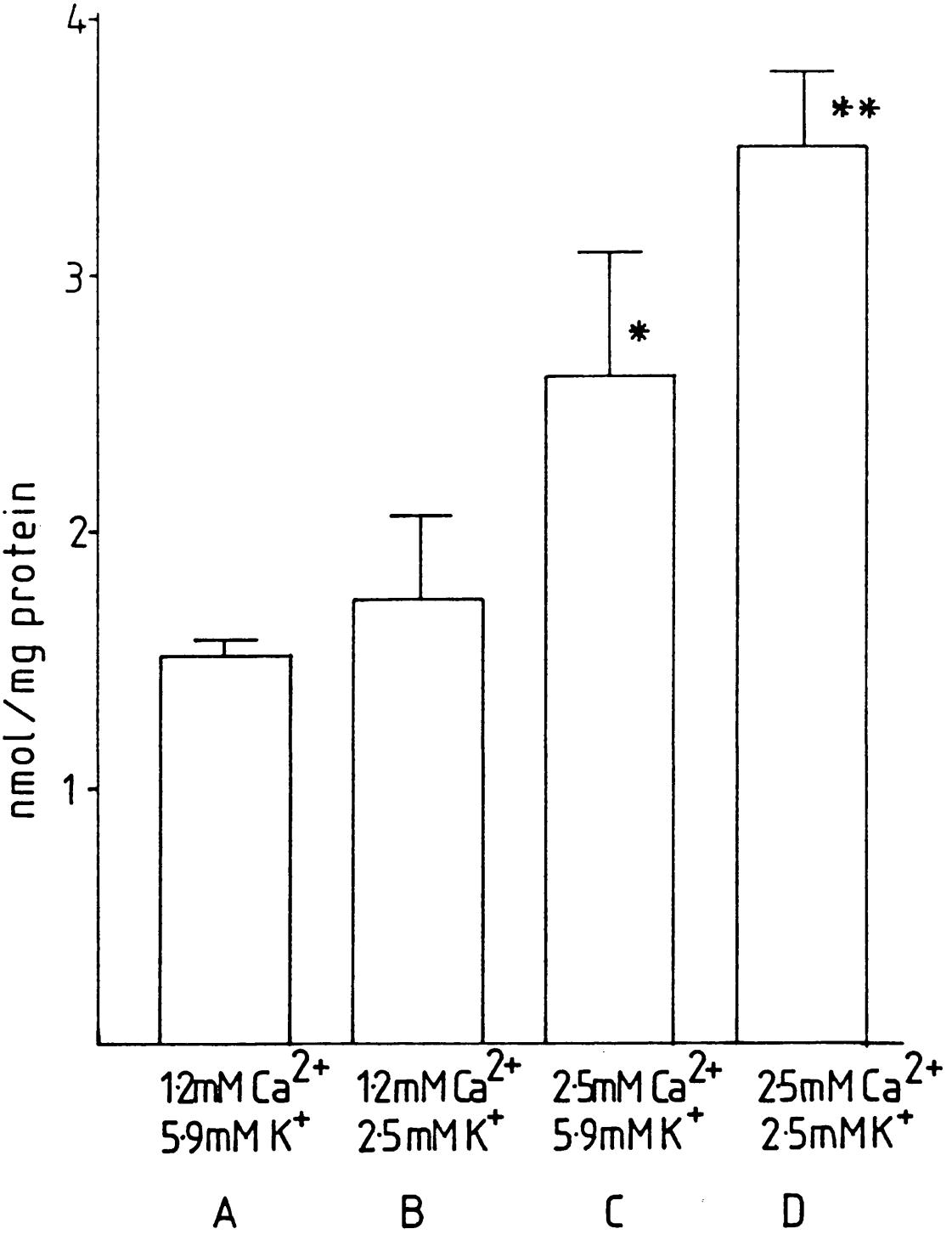


FIG 19. CHANGES IN LPC CONCENTRATION IN CONTROLS WITH DIFFERENT IONIC COMPOSITION BUFFERS. MEAN +/- SEM.

A: n = 12. B-D:n = 4.

* $p \leq 0.01$ vs A ** $p \leq 0.01$ vs B.

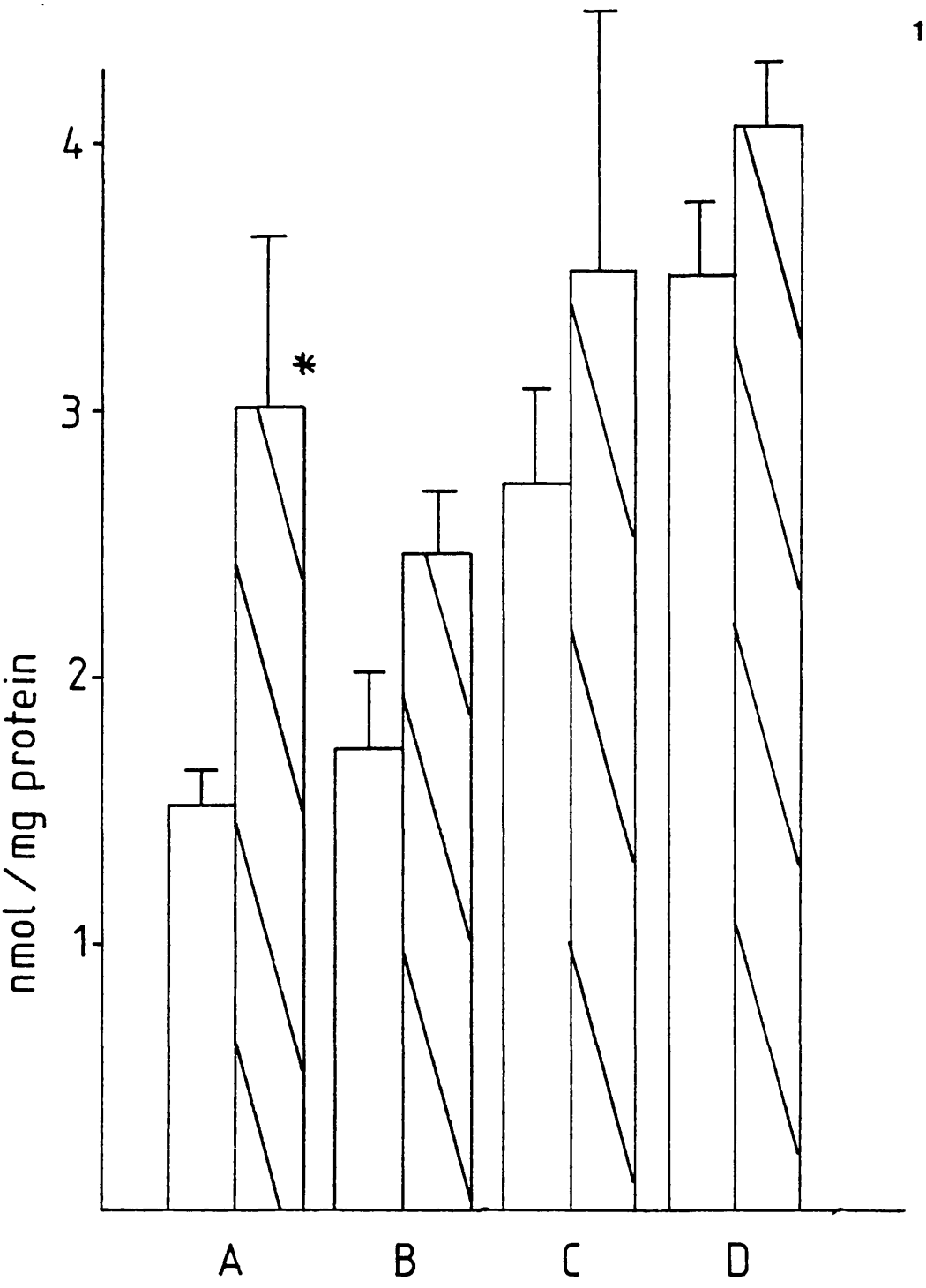


FIG 20. LPC CONCENTRATIONS FOLLOWING 20 MINUTES CORONARY ARTERY LIGATION WITH DIFFERENT IONIC COMPOSITION BUFFERS. A = 1.2mM Ca^{2+}, 5.9mM K^{+}.
B = 1.2mM Ca^{2+}, 2.5mM K^{+}.
C = 2.5mM Ca^{2+}, 5.9mM K^{+}.
D = 2.5mM Ca^{2+}, 2.5mM K^{+}.

MEAN +/- SEM. A: n = 12. B-D: n = 4.

□ SHAM LIGATED. ▨ 20 MINUTES LIGATED.

* $p \leq 0.05$ vs SHAM LIGATED.

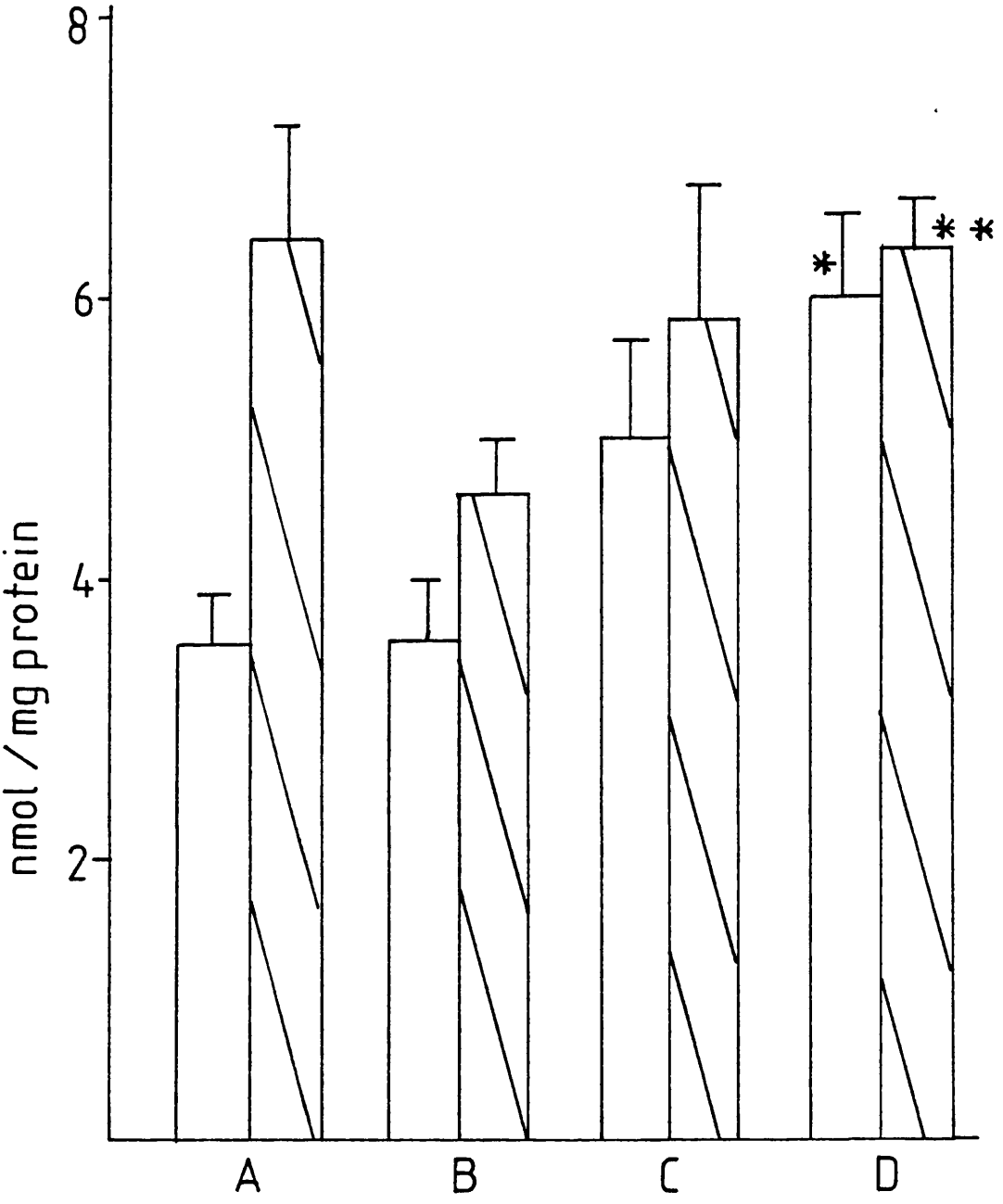


FIG 21. SUM OF LPE AND LPC CONCENTRATIONS FOLLOWING CORONARY ARTERY LIGATION (20 MINS.) WITH DIFFERENT IONIC COMPOSITION BUFFERS. MEAN +/-SEM.

A = 1.2mM Ca^{2+}, 5.9mM K^{+}.
B = 1.2mM Ca^{2+}, 2.5mM K^{+}.
C = 2.5mM Ca^{2+}, 5.9mM K^{+}.
D = 2.5mM Ca^{2+}, 2.5mM K^{+}.

A : n = 12. B-D : n = 4. * $p \leq 0.02$ vs B sham.
** $p \leq 0.02$ vs B 20 min LIGATED.

□ SHAM LIGATED. ▨ CORONARY ARTERY LIGATED.

RESULTS

8 EFFECT OF LYSOPHOSPHOLIPIDS AND RELATED COMPOUNDS IN THE ANAESTHETIZED CAT

Although LPC has been shown to produce arrhythmias in isolated hearts (section 1.5 (4)) there are no reports of its effects *in vivo*. Two modes of administration to the heart were investigated, direct infusion into the ventricular wall and into the coronary circulation.

Palmitoylcarnitine, an amphiphile with similar detergent properties to LPC, and glycerophosphorylcholine, a metabolite of LPC, were also used for comparison.

8.1 Infusion of lysophosphatidylcholine and palmitoylcarnitine into the left ventricular wall of the anaesthetized cat

i) Lysophosphatidylcholine, infused at 1ml/hr into the left ventricular wall in concentrations of 20-2000μM, produced no significant change in the heart rate or blood pressure of the anaesthetized cat (FIG 22). No change in the lead II E.C.G. was seen.

ii) Palmitoylcarnitine, infused at 1ml/hr into the left ventricular wall in concentrations of 20-1000μM, also produced no significant changes in heart rate or blood pressure of the anaesthetized cat (FIG 23). No change in lead II E.C.G. was seen.

iii) Noradrenaline, which has been shown by Podzuweit (1982) to cause arrhythmias when infused into the left ventricular wall of the pig *in vivo*, did cause arrhythmias when infused at 1ml/hr at a concentration of 10μM into the *in vivo* cat heart, producing ventricular tachycardia within 1 minute of the start of the infusion. This was reversible, the heart returning to normal rhythm approximately 6 minutes after the noradrenaline infusion was replaced with one of Evans Blue dye (0.2mg/ml 0.9% saline), and reproducible, with ventricular tachycardia recommencing 1 minute following a second noradrenaline infusion. Heart rate was therefore increased on noradrenaline infusion but the blood pressure did not change (FIG 24), presumably due to reflex compensatory mechanisms . Heart rate returned to basal levels when the noradrenaline infusion was replaced with dye in saline.

Thus, unlike 10μM noradrenaline, LPC and PAL did not produce arrhythmias when infused directly into the left ventricular wall.

8.2 Infusion of lysophosphatidylcholine, palmitoylcarnitine and glycerophosphorylcholine into the left anterior descending coronary artery of the anaesthetized cat

i) Lysophosphatidylcholine

Infusion of LPC at final blood concentrations of 20

to 100μM via the left anterior descending coronary artery caused no change in blood pressure, heart rate or lead II E.C.G..

300μM LPC was capable of producing a small number of arrhythmias with peak incidence at 14 to 17 minutes after the start of the infusion.

500μM LPC produced severe arrhythmias commencing within 5 to 10 minutes after the start of the infusion. In 2 out of 3 cats this led to ventricular fibrillation and death at 22-26 minutes. Some increase in T-wave amplitude was seen prior to the onset of arrhythmias. A typical course of arrhythmia development is shown in FIG 25.

In the case where the cat survived the 500μM LPC infusion with, few arrhythmias being produced, the coronary artery was ligated 35 minutes after the beginning of the LPC infusion. This produced ventricular fibrillation and death within 3 minutes, something that was not seen in any of the experiments where only coronary artery ligation was performed (section 8.3). LPC may be producing membrane damage which makes the heart more susceptible to fibrillation on subsequent coronary artery ligation. The possibility exists that endothelial damage may be necessary for the production of arrhythmias by other potential arrhythmogens.

ii) Palmitoylcarnitine

Infusion of PAL at final blood concentrations of 10-20μM produced no arrhythmias or changes in blood pressure and heart rate. Changes in the lead II E.C.G. did however take place, and were not reversible when the PAL infusion was replaced with one of dye in saline (FIG 26).

PAL, at concentrations of 30-40μM did produce a small number of arrhythmias and a short duration of ventricular tachycardia, with changes in the E.C.G. similar to those in FIG 26.

50-75μM PAL produced severe arrhythmias with onset at 6-10 minutes after the start of the infusion leading to ventricular fibrillation and death in 2 out of 3 cases at 18 to 21 minutes. A typical course of arrhythmia development is shown in FIG 27. ST elevation was pronounced before the onset of arrhythmias.

Thus like LPC, PAL was capable of producing arrhythmias when infused directly into the coronary circulation.

iii) Glycerophosphorylcholine

Infusion of GPC at a final blood concentration of 400μM, a concentration sufficient to cause arrhythmias with LPC and PAL, produced no arrhythmias or changes in heart rate and blood pressure.

Initial experiments had shown a large immediate increase in heart rate and blood pressure on infusion of GPC but this was found to be due to cadmium remaining in

the GPC solution (see method of GPC preparation section 3.2) as these changes could be mimicked by a solution of cadmium chloride. Cadmium chloride, at final blood concentrations of 200-300μM gave increased heart rate (+ 20 bpm) and blood pressure (+ 20mm Hg) with 400μM $CdCl_2$ producing arrhythmias, and in one case ventricular fibrillation and death.

8.3 Effect of coronary artery ligation on the production of arrhythmias and on temperature in the left ventricular wall of the anaesthetized cat

In order to compare the effects of LPC, PAL and GPC on arrhythmogenesis with arrhythmias produced by coronary artery ligation some experiments were carried out whereby the coronary artery was ligated and arrhythmias monitored. In some of these the temperature in the left and right ventricular walls was monitored to see if this could be used as a marker of ischaemia.

Coronary artery ligation produced arrhythmias in all 6 animals. In 3 this led to ventricular fibrillation and death (mean number of extrasystoles = 554 +/- 262) with fibrillation occuring at 26.6 +/- 6.7 minutes post ligation. The remaining animals recovered from arrhythmias (mean number of extrasystoles = 415 +/- 88) and survived to one hour post ligation by which time the heart had returned to normal rhythm. Those cats developing ventricular fibrillation after coronary artery

ligation did so at a similar time to those given LPC or PAL infusion. This was also the time of highest ectopic activity in cats that survived one hour of coronary artery ligation. Lead II E.C.G. changes were seen after coronary artery ligation (FIG 28) as were changes in blood pressure and heart rate (FIG 29).

After coronary artery ligation the temperature in the left ventricular wall decreased while that in the non-ischaemic area remained the same (FIG 30). A reduced temperature was therefore taken to be an indicator of ischaemia. On occasions where no fall in temperature was observed, or where an initial fall was followed by an increase back to the initial value, it was found that there was no defined ischaemic area as judged by i.v. dye infusion. In these cases there was probably a considerable amount of collateral flow.

8.4 Effect of palmitoylcarnitine and streptokinase infusion into the coronary artery on the temperature in the left ventricular wall of the anaesthetized cat

As infusion of palmitoylcarnitine into the coronary artery produced ST elevation experiments were carried out to see if this was indicative of ischaemia by monitoring left ventricular wall temperature and examining the ability of streptokinase to reverse any of these changes.

Infusion of 20μM palmitoylcarnitine (final blood

concentration), which did not produce arrhythmias, produced ST elevation and a decrease in temperature in the left ventricular wall. This temperature fall was not reversed when the palmitoylcarnitine infusion was replaced with one of dye in saline but was reversed by an infusion of streptokinase (170U/min) (FIG 31).

Palmitoylcarnitine thus appeared to cause ischaemia, as evidenced by the fall in temperature in the left ventricular wall. It is unlikely to be caused by vasoconstriction as it was not reversed by dye in saline but was reversed by streptokinase infusion. Palmitoylcarnitine therefore appears to be producing ischaemia by some effect on haemostasis in the coronary arteries.

8.5 Effect of lysophosphatidylcholine infusion into the coronary artery of the anaesthetized cat on the left ventricular wall temperature

As palmitoylcarnitine appeared to produce ischaemia the effect of LPC on left ventricular wall temperature was monitored to check whether it produced the same effects as PAL.

Infusion of lysophosphatidylcholine at a final blood concentration of 300μM produced no change in the temperature in the left ventricular wall. Correct positioning of the probes was validated by the decrease in temperature seen on coronary artery ligation and by

dye infusion i.v. to visualise the ischaemic area.

8.6 Effect of lysophosphatidylcholine and palmitoylcarnitine on rat and rabbit platelet aggregation

PAL appeared to produce ischaemia when infused into the coronary artery of the anaesthetized cat. This may be mediated by effects on platelet aggregation therefore the effects of both PAL and LPC on this were studied.

Neither PAL nor LPC stimulated platelet aggregation. No difference was seen between rat and rabbit platelets in their response. The effect of intracoronary PAL in producing ischaemia in the cat which was reversible by streptokinase is therefore unlikely to be due to a direct effect on platelets. This does not exclude the possibility of platelet behaviour being modified in whole blood *in vivo* as opposed to platelet rich plasma (Harrison, Pollock, Steiner and Weisblatt 1985), or an indirect effect with other components of the blood.

8 DISCUSSION

EFFECT OF LYSOPHOSPHOLIPIDS AND RELATED COMPOUNDS IN THE ANAESTHETIZED CAT

Infusion of lysophosphatidylcholine and palmitoylcarnitine into the left ventricular wall of the anaesthetized cat

No effects of LPC were seen when it was infused directly into the left ventricular wall at concentrations up to 2mM. This surpasses concentrations found in ischaemic tissue during arrhythmias of 1.65mM (Corr and Snyder et.al. 1982) and 1.2mM (calculated from results in section 7.2 at 20 minutes of ischaemia, LPC + LPE = 6.22 x 10^{-9} mol/mg protein, assuming 193 mg protein / g wet weight and 1ml = 1 g wet weight then LPC + LPE = 1.2mM) and those shown to produce electrophysiological changes in tissue when added exogenously (Nakaya and Ozaki et.al. 1984, Sawicki and Arnsdorf 1985).

Although albumin binding of LPC would be expected to occur this would also occur with LPC generated during ischaemia and therefore the same proportion of free LPC should exist. It is possible that endogenously produced LPC will only be slowly washed out of the membranes and therefore the effective concentration in the membrane is relatively high as the interstitial fluid has a lower albumin concentration than blood (Hülsmann, Stam and

Lamers 1985). Protein concentration in the interstitial fluid has been estimated to be 0.2mM as opposed to 1mM in plasma although a ratio of albumin concentration of 0.85 in the lymph in comparison to the plasma has been measured (Laine and Granger 1985). There is thought to be free movement of LPC between the vascular and extracellular space and labelled LPC that has been previously taken up by the perfused rat heart can be eluted by perfusion with a protein containing medium (Stein and Stein 1965) therefore infused LPC may be eluted rapidly by the blood and diluted to an ineffective concentration before incorporation into the cellular membranes is able to occur.

PAL, which has similar detergent-like properties to LPC because of structural similarities, also produced no effects when infused into the left ventricular wall. This may again demonstrate the rapid dilution of the ampniphile, precluding its incorporation into the cell membrane. PAL accumulates in the sarcolemma during ischaemia and may bind selectively to membrane proteins rather than distribute within the lipid bilayer (Knabb, Saffitz, Corr and Sobel 1986). Its effects during endogenous generation in ischaemia may differ from those when added exogenously and may result from effects on cellular metabolic processes in the former case, being an intermediate in fatty acid oxidation, rather than non-specific detergent effects in the latter.

Noradrenaline was shown to produce reversible, reproducible ventricular tachycardia, in common with the results of Podzuweit (1982) in the pig. Albumin binding of noradrenaline does not occur and thus noradrenaline is unlikely to be removed as avidly from the interstitial space as LPC or PAL. It is possible that LPC or PAL need to incorporate into a larger area of tissue to produce electrophysiological effects than does noradrenaline due to the latters specific receptor mediated effects as opposed to the possibly non-specific depression of membrane conductance produced by LPC or PAL (Clarkson and Ten Eick 1983). The effect of noradrenaline is only temporary. When the infusion is stopped uptake of noradrenaline into cells occurs thus its concentration is rapidly reduced. Diffusion away from the nerve terminal will also occur.

SUMMARY: LPC and PAL do not produce any effects when infused into the left ventricular wall, possibly due to binding to albumin and washout from the interstitial space. It is also possible that they need to affect a large area of tissue for any electrophysiological effects to be seen.

Infusion of lysophosphatidylcholine into the coronary circulation

Unlike infusions into the ventricular wall, intracoronary LPC did produce concentration dependent arrhythmias.

At low final blood concentrations of LPC (20-100μM) no arrhythmias were seen, however at a final blood concentration of 500μM (assuming a blood flow of 4ml/min) severe arrhythmias were seen leading to ventricular fibrillation and death. This concentration is higher than that producing arrhythmias when perfused through isolated hearts i.e. 50 - 100μM in the isolated hamster heart (Man and Choy 1982, Man, Wong and Choy 1983), 20μM (Man and Lederman 1985) and 100μM (Bergmann et.al. 1981) in the isolated rabbit heart. However all these values were for albumin free LPC. As the free concentration of LPC appears to be tne important factor in initiating both arrhythmias (Man and Choy 1982) and electrophysiological changes (Corr and Snyder et.al. 1981) rather than the total concentration, the binding of LPC by the blood proteins is likely to be of importance. Blood albumin concentration is approximately 500 - 600μM (Lehninger 1975) and as LPC binding is thought to saturate at a ratio of 1.4 : 1 (Klopfenstein 1969) the binding capacity of albumin is likely to have reached saturation on infusion of 500μM LPC because the endogenous venous

plasma LPC concentration has been shown to average 370 +/- 20 μM in the anaesthetized cat (Snyder et.al. 1981) and thus the albumin binding capacity will already be partly saturated before any LPC is infused.

LPC can be taken up by the isolated rat heart while complexed to albumin (Stein and Stein 1965), this uptake being dependent on the LPC/albumin ratio and was high at an LPC/albumin ratio of less than 1. LPC uptake has also been shown in the in vivo dog heart (Riemersma and Michorowski 1983).

At an infused LPC concentration of 500μM, albumin binding of LPC is therefore likely to be saturated. If a basal concentration of LPC of 370μM, an albumin concentration of 500μM and a saturation binding of 1.4 LPC : 1 albumin are assumed, a final free LPC concentration of approximately 170μM would result on infusion of 500μM (final blood concentration) LPC. This is in the same range as the free LPC concentration producing arrhythmias in isolated hearts. At this concentration there is likely to be considerable uptake of LPC which is not confined to the vascular tissue and if this is not reacylated (as only 15% of LPC taken up was seen to be by Stein and Stein (1965)) incorporation into the cellular membranes will occur. This may lead to changes in ion conductances, membrane enzyme activities and characteristics which can lead to the production of arrhythmias.

It has been suggested that lysophospholipids in ischaemia have a vascular or endothelial origin with their release into the coronary circulation (Stam and Hülsmann 1981), thus intracoronary infusion of LPC may mimic this release and the subsequent arrhythmias may be due to its incorporation into the membranes of both vascular cells and myocytes. If this is the mechanism by which LPC generated during ischaemia exerts its arrhythmogenic effects lower concentrations are likely to be effective in ischaemic, as opposed to normoxic, tissue as the reduced pH seen in ischaemia potentiates the electrophysiological effects of LPC without increasing its incorporation (Gross et.al. 1982) and a reduced pH reduces albumin binding (Kurien and Olivier 1970).

Although LPC incorporation into the sarcolemmal and mitochondrial membranes is probably the ultimate cause of membrane dysfunction and arrhythmogenic effects both endogenously generated lysophospholipids and lysophospholipids generated at one site and subsequently incorporated into other cells may play a role.

Prior to the development of arrhythmias during infusion of 500μM LPC there were no gross changes in the lead II E.C.G. and no ST elevation (a characteristic of ischaemia) was seen. An increase in T-wave amplitude was seen prior to the development of arrhythmias. This is in agreement with the findings of Bergmann et.al. (1981) in the isolated rabbit heart where little change was seen in

the epicardial electrogram apart from some conduction delay (not seen in the anaesthetized cat) and changes in T-wave amplitude and morphology. The increase in T-wave has also been shown in anaesthetized cats following coronary artery occlusion, in conjunction with ST elevation (Spath, Lane and Lefer 1974).

The time course of the LPC induced arrhythmias was similar to that of coronary artery ligation induced arrhythmias in the anaesthetized cat. This may be due to initial rapid incorporation of LPC inducing the same electrophysiological changes seen in ischaemia which, once induced, lead to the production of arrhythmias and thus once LPC production (in ischaemia) and incorporation (in LPC infusion) have passed a certain level the same mechanisms lead to the development of arrhythmias. Further infusion of LPC may not lead to greater effects as an all or none response may be operating with a certain membrane concentration of LPC being needed to elicit effects.

Glycerophosphorylcholine did not produce any arrhythmias, at a final blood concentration of 400μM. It is thus unlikely that LPC is exerting its effects by breakdown into GPC. The latter is not amphiphilic and does not have detergent like actions thus lending support to the hypothesis that the effects of LPC are due to these properties. This is in agreement with the lack of effect of GPC and free fatty acids on

electrophysiological characteristics of canine Purkinje fibres (Corr et.al. 1979).

SUMMARY: LPC, infused into the coronary artery, is therefore capable of producing arrhythmias in the anaesthetized cat. These arrhythmias follow the same time course as coronary artery ligation induced arrhythmias and may be ultimately caused by electrophysiological changes produced by incorporation of LPC into the sarcolemma. It is also possible that LPC is causing damage to the vascular tissue, in particular to the endothelial cells, releasing potential arrhythmogens and by reducing their ability to act as a permeability barrier this could allow other potential arrhythmogens to enter the tissue from the circulation.

Infusion of palmitoylcarnitine into the coronary circulation

Like LPC, PAL produced arrhythmias when given intracoronary, but at lower concentrations than did LPC.

Low concentrations of PAL that did not produce arrhythmias did produce changes in the lead II E.C.G. indicative of ischaemia, i.e. ST elevation. This ST elevation increased as the PAL concentration was increased and was not reversed by replacing the PAL infusion with one of dye in saline therefore it is unlikely to be due to vasoconstriction.

In cases where PAL produced arrhythmias the final blood concentration of PAL was 50 - 75 μM. This concentration is approximately a tenth of that of LPC needed to produce the same arrhythmogenic effects, therefore if non-specific detergent effects of LPC are important in producing arrhythmias some other mechanism must also apply for PAL. It is possible that binding of PAL by serum albumin does not have as high an affinity or capacity and thus the same free concentration of this amphiphile may be achieved at different total concentrations. It seems unlikely that this would account for a 10 fold difference.

PAL has similar amphiphilic properties to LPC therefore its incorporation into the tissue is likely to occur and this could mimic production and accumulation of PAL during ischaemia. Preferential accumulation into the rat myocyte sarcolemma during hypoxia has been demonstrated (Knabb et.al. 1986). Such accumulation was thought to cause electrophysiological changes similar to those induced by LPC. Differences in the membrane dynamics of sarcolemma were seen when LPC or PAL were incorporated into isolated canine sarcolemma thus although LPC and PAL are both amphiphiles there are differences between their effects on membranes (Fink and Gross 1984).

Infusion of PAL at final blood concentrations producing ST elevation also produced a decrease in

temperature of the left ventricular wall. This is a further indication of the development of ischaemia within the tissue as this fall in temperature could also be produced by coronary artery ligation. The drop in temperature produced by PAL could be reversed by infusion of streptokinase (170U/min).

Streptokinase, a single chain polypeptide of molecular weight 48,000, has thrombolytic effects and has been used clinically for the treatment of thrombosis (Kennedy, Ritchie, Davis, Stadius, Maynard and Fritz 1985, Olson, Butman, Piters, Gardin, Lejons, Jones, Chilazu, Kumar and Colombo 1986). It acts on the plasminogen-plasmin system by binding to plasminogen, producing an active site in the latter which can activate a further molecule of plasminogen to plasmin (Sarnama, Szwarcer, Conard and Horellou 1985). Plasmin is a proteolytic enzyme that converts fibrin polymer to soluble components and therefore streptokinase acts on a preformed clot to break down the fibrin polymer. There is not a close correlation between administered dose and the response due to the mechanism of action, high doses binding all the free plasminogen and leaving none free to be activated by proteolytic cleavage (Johnson and MacCarty 1959).

The ability of streptokinase to reverse the drop in temperature seen on PAL infusion suggests that PAL causes the production of intracoronary occlusion. As a drop in

temperature and ST elevation were not seen on LPC infusion this represents an important difference between the action of the two amphiphiles, with PAL apparently causing this formation whilst LPC did not. The time course of the PAL induced arrhythmias was again similar to those produced by coronary artery ligation and by 500μM LPC infusion, thus this may be a result of the ischaemia produced by PAL leading to the same mechanism of arrhythmia production as occurs following coronary artery ligation.

PAL could produce a reduction in coronary flow by various mechanisms. As no effect of LPC or PAL was seen on rat or rabbit platelet aggregation it is unlikely that PAL was acting directly on platelets to cause aggregation although it is still possible that a species difference may be present between rat, rabbit and cat platelets. It has been shown that platelet aggregation is associated with the level of haematocrit (Harrison, Pollock, Steiner and Weisblatt 1985). This was thought to be due to the increased number of platelet collisions or increased ADP release from damaged erythrocytes at high haematocrit. Therefore <u>in vivo</u> platelet aggregation may occur due to release of ADP from damaged erythrocytes. PAL may cause haemolysis as lysis of erythrocytes at amphiphile concentrations exceeding the critical micelle concentration has been shown (Bergmann et.al. 1981). It would be expected that LPC would also produce these

effects unless binding to albumin or uptake into the heart is more extensive, leading to a reduced effective LPC concentration.

PAL could also be interacting with the vascular endothelium and causing release of a pro-aggregatory substance. There may also be a vasoconstrictor component potentiating the effects of any thrombi, as PAL has been shown to contract aortic rings in a concentration dependent manner, although this was not dependent on the presence of endothelium, and can also antagonise the effects of endothelium dependent relaxant factor (Bigaud and Spedding 1986).

SUMMARY: PAL, when infused into the coronary circulation, produces arrhythmias which have a similar time course to those initiated by intracoronary LPC and by coronary artery ligation. The effect of PAL differs from that of LPC in that the former is effective at one tenth the concentration of LPC and appears to produce ischaemia. The effect of PAL in producing ischaemia may be due to an effect on the coronary vasculature with release of a pro-aggregatory substance coupled with vasoconstriction, but is unlikely to be due to a direct effect on platelet aggregation.

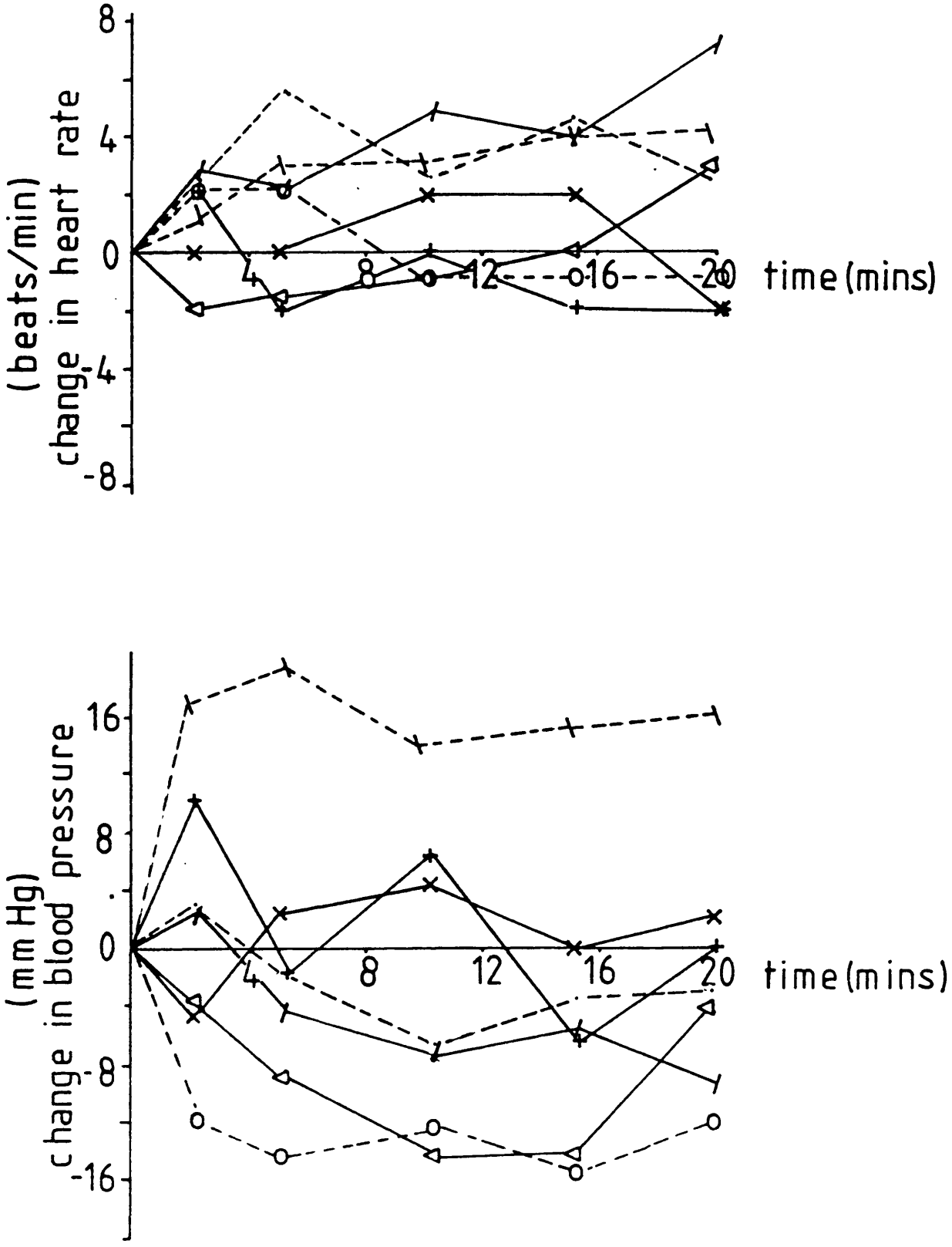


FIG 22. CHANGES IN BLOOD PRESSURE AND HEART RATE OF THE ANAESTHETIZED CAT ON INFUSION OF LPC INTO THE LEFT VENTRICULAR WALL.
× 20uM, O 50uM, ◁ 100uM, \ 200uM, / 500uM, • 1000uM,
+ 2000uM. MEAN OF 1 - 3.

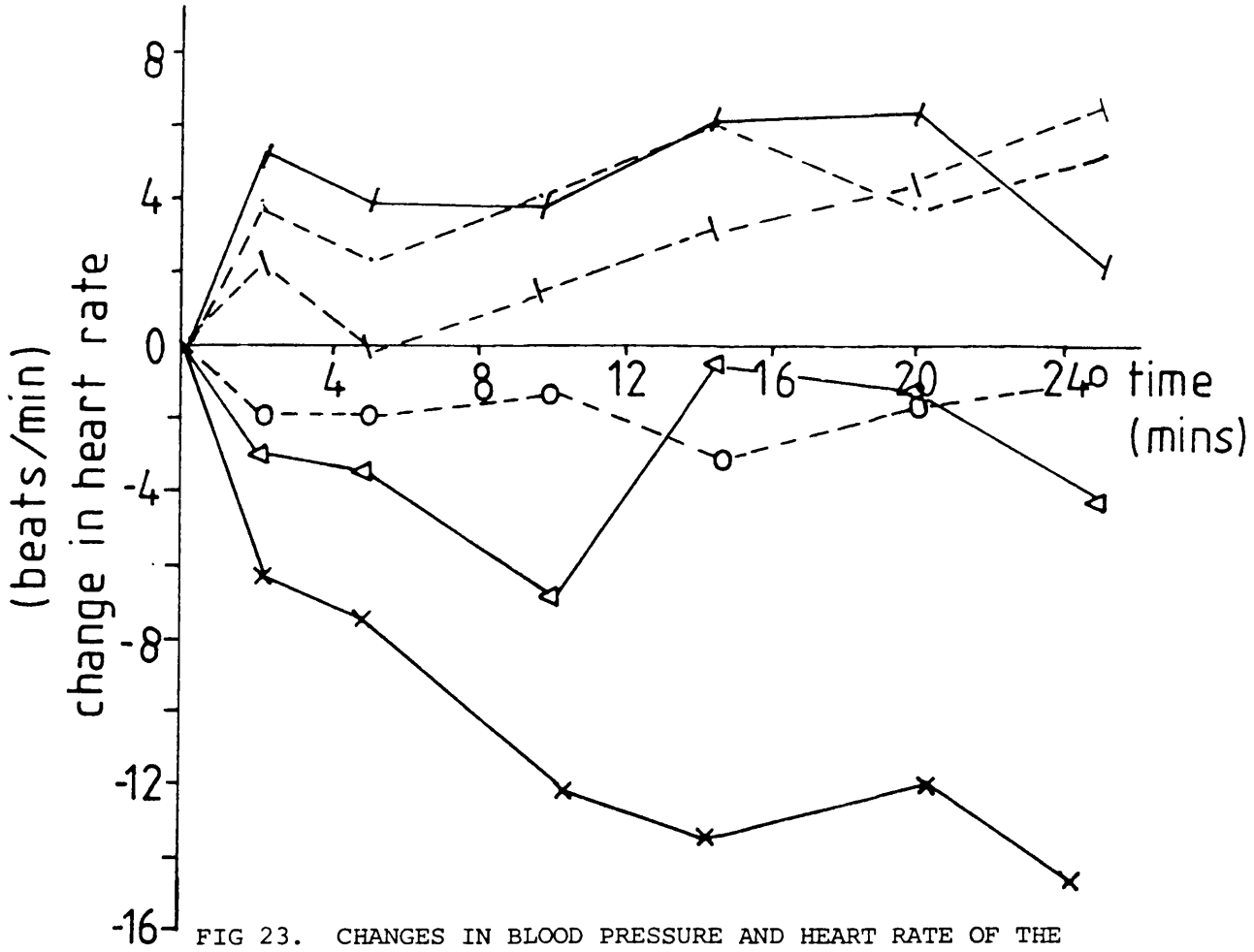
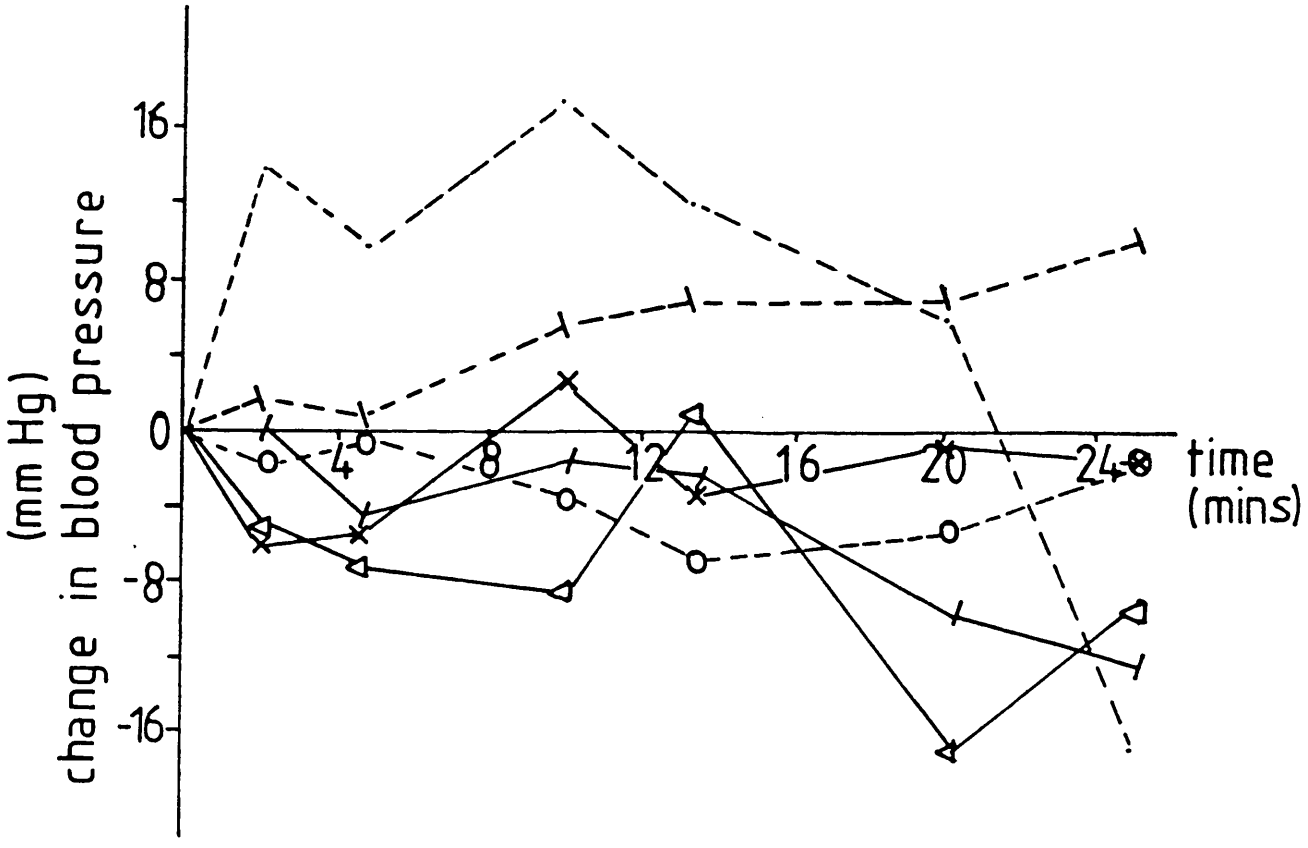


FIG 23. CHANGES IN BLOOD PRESSURE AND HEART RATE OF THE ANAESTHETIZED CAT ON INFUSION OF PAL INTO THE LEFT VENTRICULAR WALL. MEAN OF 2.
× 20uM, O 50uM, ◁ 100uM, \ 200uM, / 500uM, · 1000uM,



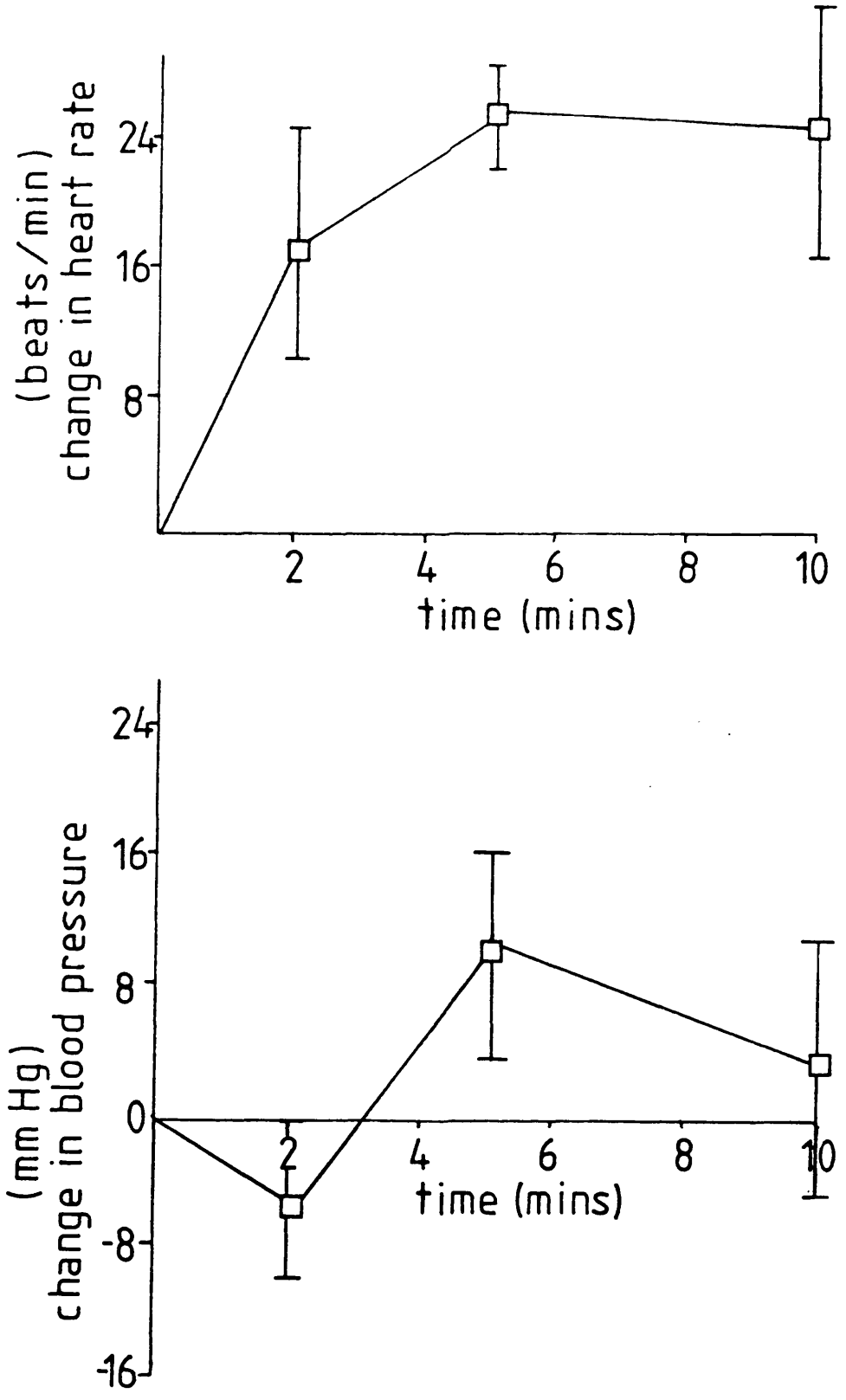


FIG 24. CHANGES IN BLOOD PRESSURE AND HEART RATE OF THE ANAESTHETIZED CAT ON INFUSION OF 10μM NORADRENALINE INTO THE LEFT VENTRICULAR WALL. n = 5. MEAN +/- SEM.

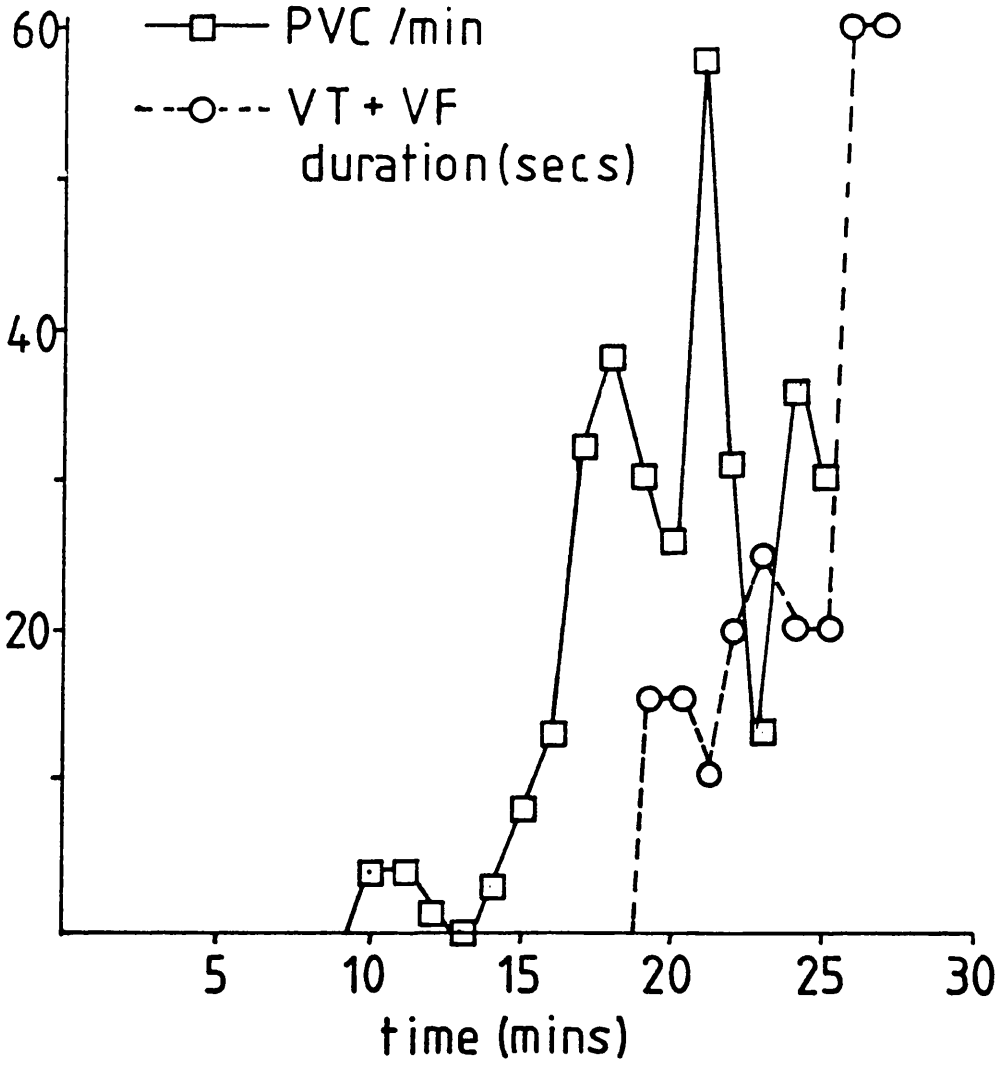


FIG 25. THE PRODUCTION OF ARRHYTHMIAS BY INFUSION OF 500μM LPC (FINAL BLOOD CONCENTRATION) INTO THE CORONARY ARTERY OF THE ANAESTHETIZED CAT. VT = VENTRICULAR TACHYCARDIA, VT = VENTRICULAR FIBRILLATION, PVC = PREMATURE VENTRICULAR CONTRACTIONS.

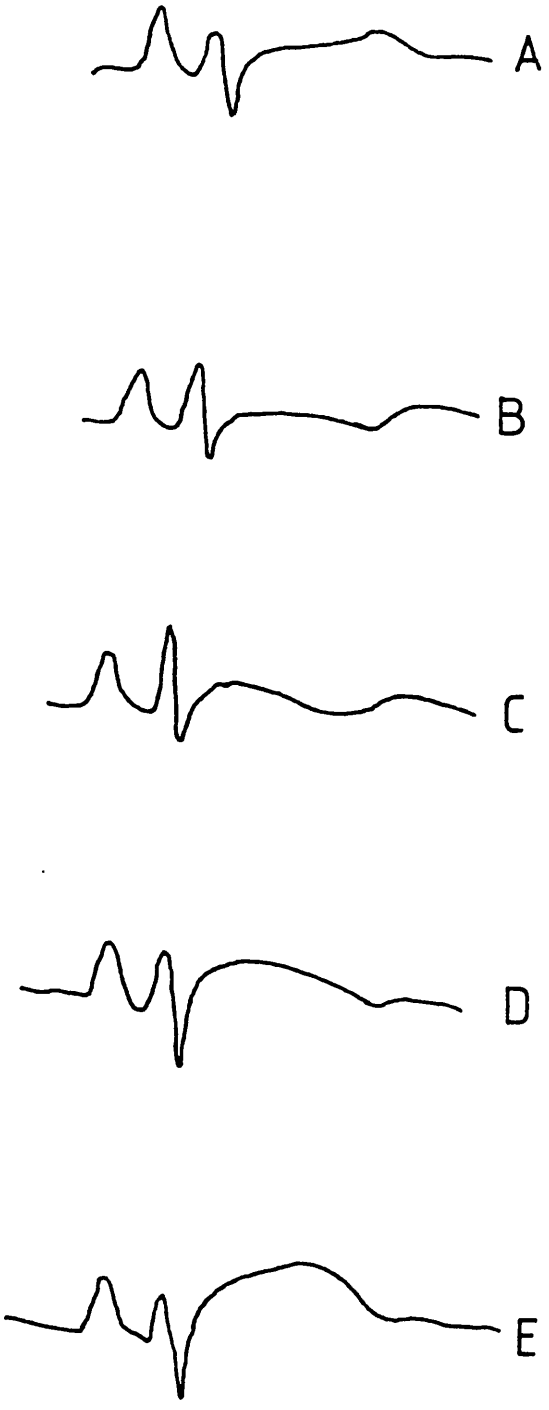
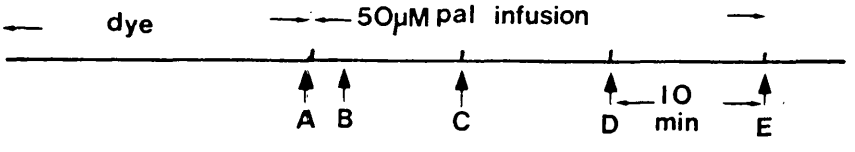


FIG 26. LEAD II E.C.G. ON INFUSION OF INTRACORONARY PALMITOYL-CARNITINE IN THE ANAESTHETIZED CAT.



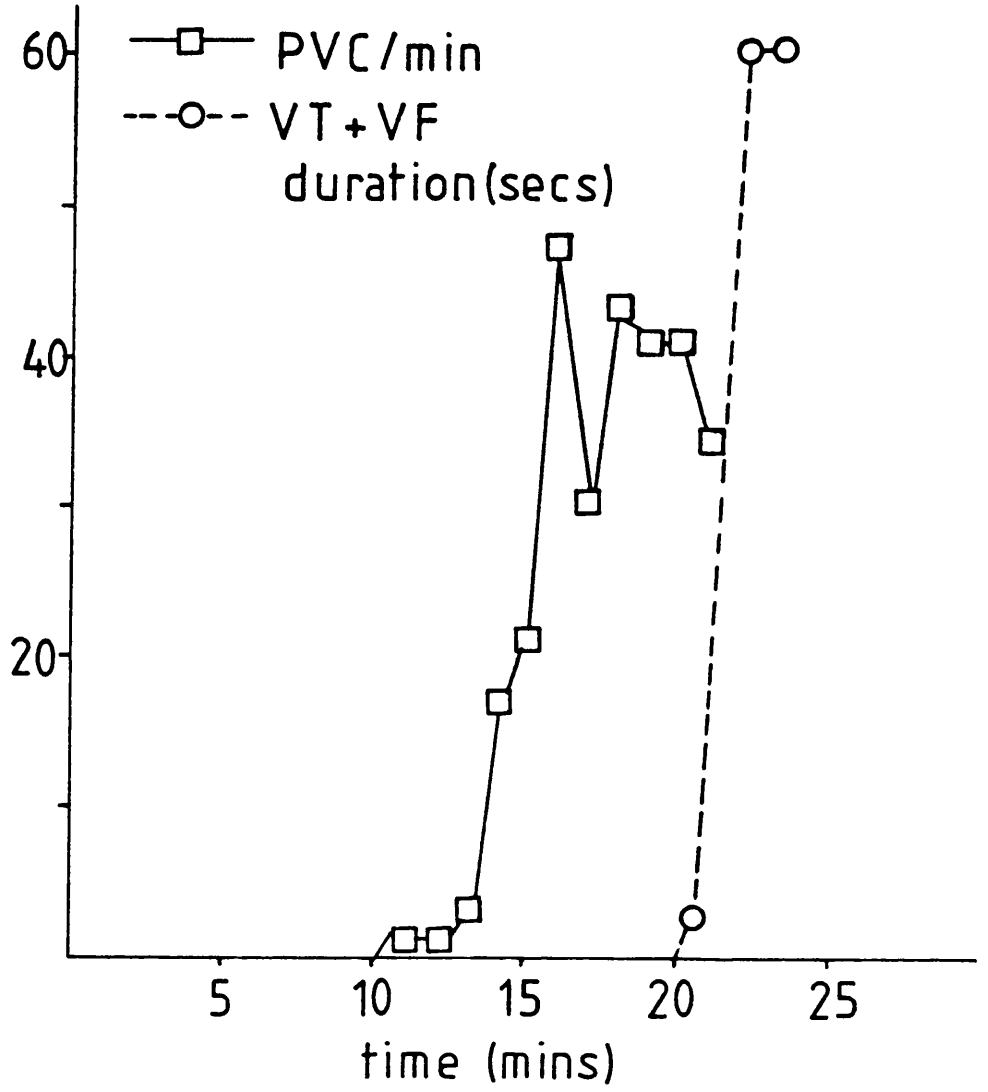


FIG 27. THE PRODUCTION OF ARRHYTHMIAS BY THE INFUSION OF 50μM PAL (FINAL BLOOD CONCENTRATION) INTO THE CORONARY ARTERY OF THE ANAESTHETIZED CAT. VT = VENTRICULAR TACHYCARDIA, VF = VENTRICULAR FIBRILLATION, PVC = PREMATURE VENTRICULAR CONTRACTIONS.

FIG 28. E.C.G. CHANGES SEEN ON LIGATION OF THE LEFT DESCENDING CORONARY ARTERY OF THE ANAESTHETIZED CAT.
A = CONTROL, B = 2 MINUTES POST LIGATION,
C = 10 MINUTES POST LIGATION.

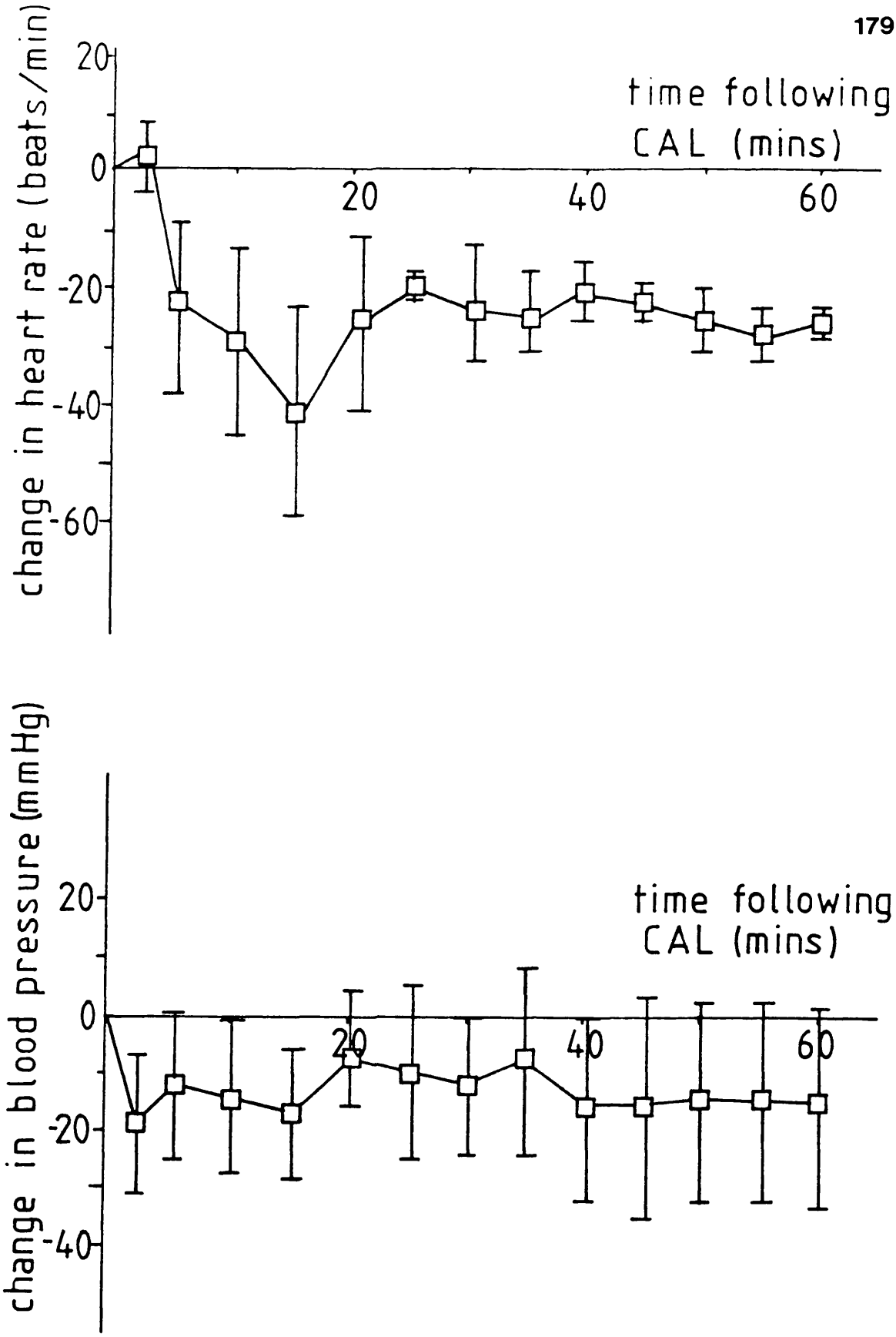


FIG 29. CHANGES IN BLOOD PRESSURE AND HEART RATE FOLLOWING CORONARY ARTERY LIGATION IN THE ANAESTHETIZED CAT. MEAN +/- SEM. 0-15 min n = 6. 20 min n = 5, 25-35 min n = 4, 40-60 min n = 3.

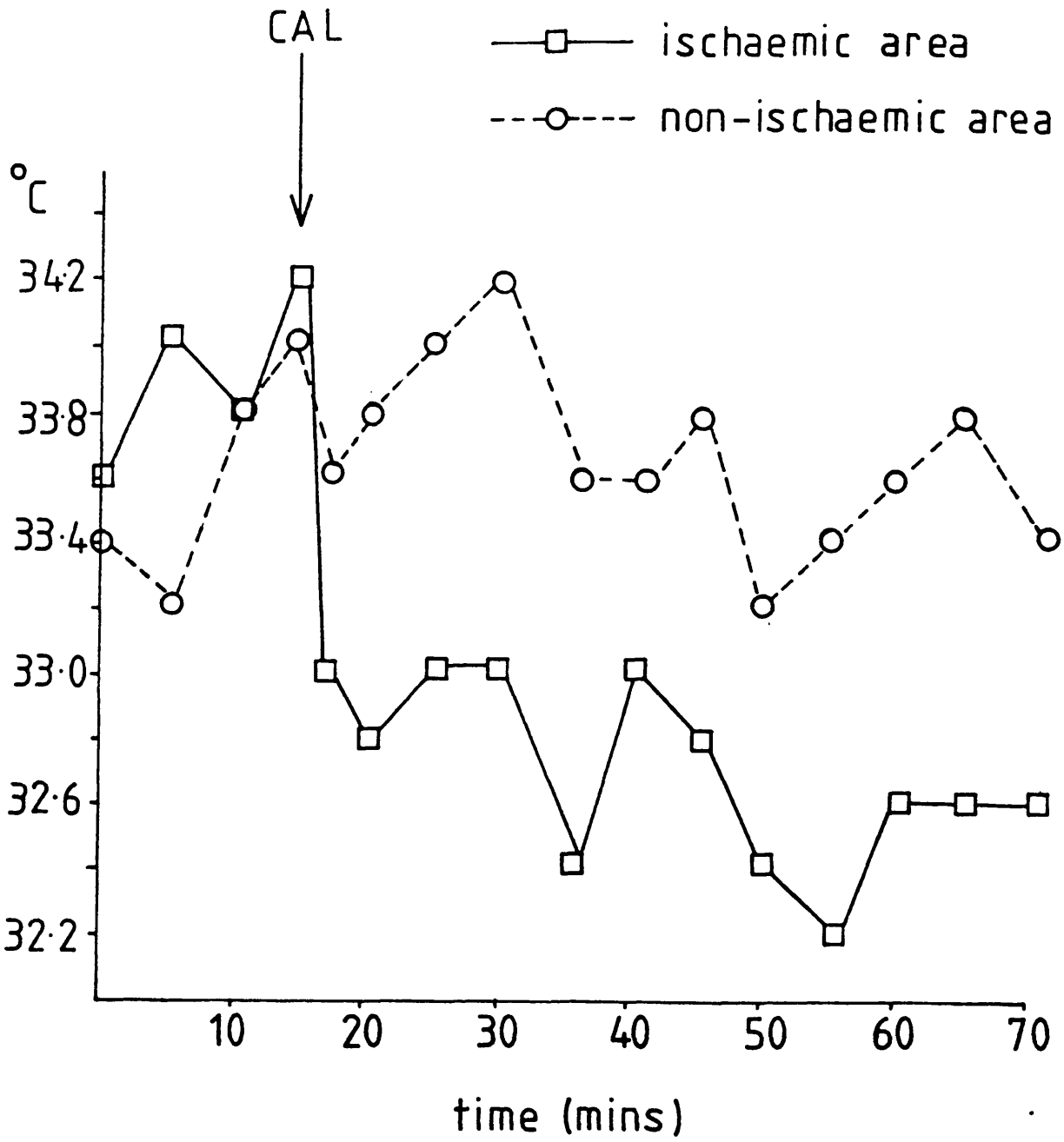


FIG 30. CHANGES IN TEMPERATURE IN THE ISCHAEMIC AND NON-ISCHAEMIC AREAS FOLLOWING CORONARY ARTERY LIGATION IN THE ANAESTHETIZED CAT. CAL = CORONARY ARTERY LIGATION.

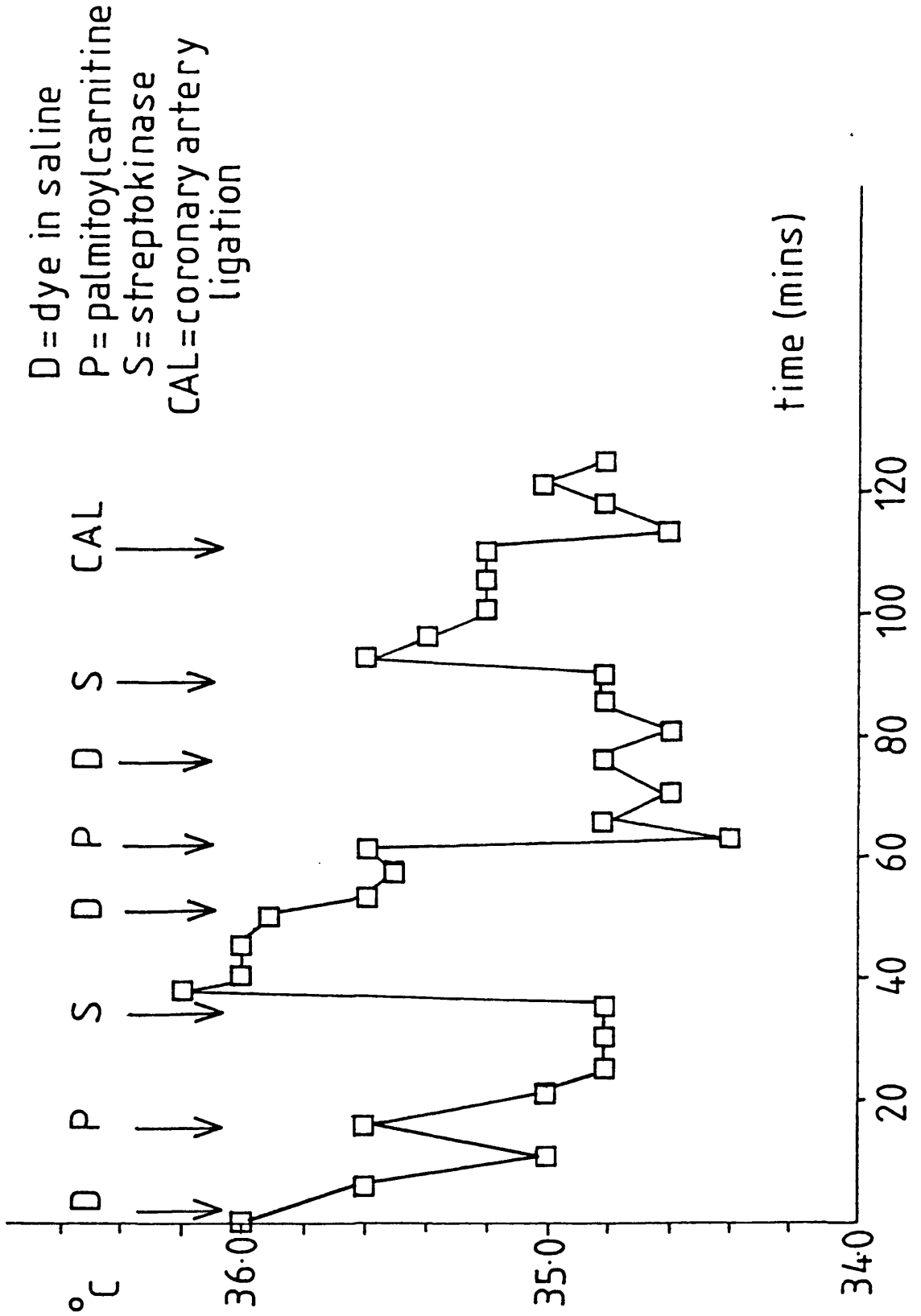


FIG 31. EFFECT OF 20μM PALMITOYLCARNITINE AND STREPTOKINASE INFUSION (170 U/min) ON THE TEMPERATURE OF THE LEFT VENTRICULAR WALL OF THE ANAESTHETIZED CAT.

RESULTS

9 MEASUREMENT OF PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART

As LPC concentration increased during ischaemia it was decided to assay phospholipase A_2 activity to see if an increase in its activity could account for the rise in LPC.

9.1 Characterisation of phospholipase A_2 in the homogenate of the isolated rat heart

i) Protein dependence

In the phospholipase A_2 assay the quantity of C^{14}-oleic acid released was proportional to the amount of protein added to the assay. No release of C^{14}-oleic acid was seen with varying volumes of protein homogenate that had been boiled to inactivate the enzymes (FIG 32). Thus the rate of product release is proportional to enzyme concentration.

ii) Calcium dependence

The release of C^{14}-oleic acid was dependent on calcium. Increasing the calcium concentration increased the reaction rate to a maximum at 3-5 mM Ca^{2+} (FIG 33). A calcium concentration of 5 mM was therefore chosen for future assays of phospholipase A_2 as it would prevent Ca^{2+} from being rate limiting. Some activity was seen in the presence of 10mM EDTA which may be due to binding of

calcium to the enzyme that EDTA could not displace or a calcium independent reaction. Using the direct linear plot for kinetic analysis a Km of 0.6mM for calcium was obtained.

iii) Time dependence

The release of C^{14}-oleic acid due to enzymic release from the labelled PE was linear with time until at least 45 minutes (FIG 34), therefore a 45 minute incubation period was chosen for all future experiments as it provided a compromise between rapidity of the assay and accuracy by maximisation of the quantity of C^{14}-oleic acid released.

iv) Loss of phospholipase A_2 activity on freezing

a) Samples previously assayed freshly that were reassayed after being stored at -20°C for up to 5 months were found to have retained 57.9 +/- 3.4% of their original activity (n = 9). Samples having original activity ranging from 2.39 to 11.47 x 10^{-11}mol/mg protein /hr were chosen and the activity loss appeared to be a constant percentage of the initial activity rather than a decrease to a common basal activity.

b) The loss of activity on freezing appeared to be due to an initial loss on freezing, the activity thereafter remaining constant (FIG 35), in common with the findings of Corr, Ahumada and Sobel (1982).

In tissue samples that had to be stored before assay it was concluded that some loss of activity would have occurred on the initial freezing. This loss of activity is likely to be of the same percentage in all samples and therefore allows comparison of samples. As the loss of activity seems to be due to an initial loss on freezing samples frozen for different periods of time can be compared.

9.2 Effect of coronary artery ligation on phospholipase A_2 activity in the ischaemic and non-ischaemic regions of the isolated rat heart

Sham ligated control hearts showed no difference in PLA_2 activity between the regions corresponding to the ischaemic and non-ischaemic areas in hearts subjected to coronary artery ligation.

Following 5 minutes coronary artery ligation there was no difference in the activity of PLA_2 between the ischaemic and non-ischaemic areas (FIG 36). The pH optimum was in the neutral to alkaline region, pH 7-8.

Twenty minutes following coronary artery ligation there was a significant depression of PLA_2 activity in the ischaemic region in comparison to the non-ischaemic region (FIG 37).

It would appear that as the length of ischaemia increases inhibition of PLA_2 occurs, being significant at 20 minutes post ligation when the concentrations of lyso-

phospholipids (FIG 14 + 15) and the arrhythmias are at their peak.

9.3 Mitochondrial phospholipase A_2

i) pH dependence

Mitochondrial PLA_2 showed a broad pH profile with maximal activity at pH 6-8 (FIG 38). The pH profile was similar to that seen in the whole heart homogenate (FIG 36).

ii) Calcium dependency

The activity of mitochondrial PLA_2 increased as the calcium concentration was increased, to a maximal activity at 5-7.5mM Ca^{2+} (FIG 39), a calcium concentration of 5mM being chosen for further experiments to prevent calcium being rate limiting.

9.4 Effect of coronary artery ligation on the activity of mitochondrial phospholipase A_2 in the ischaemic and non-ischaemic regions of the isolated rat heart

Following 20 minutes coronary artery ligation mitochondria isolated from the ischaemic region exhibited a depressed PLA_2 activity in comparison to those isolated from the non-ischaemic area (FIG 40). This is similar to the change in PLA_2 activity seen in the homogenate of the isolated rat heart following 20 minutes coronary artery

ligation (FIG 37).

9.5 Sarcolemmal phospholipase A_2

i) Calcium dependency

Sarcolemmal PLA_2 activity increased with calcium concentration up to a plateau at 3 to 5 mM Ca^{2+} (FIG 41). A concentration of 5mM Ca^{2+} was therefore chosen for further experiments to produce maximal activity. This is in common with the homogenate and mitochondria which were found to have similar calcium dependencies (FIGS 33 + 39).

The sarcolemmal PLA_2 showed some activity in the presence of 10mM EDTA. This may be due to high affinity binding of calcium by the sarcolemma and/or the formation of vesicles retaining calcium which is not accessible to the EDTA, or a calcium independent enzyme.

9.6 Effect of coronary artery ligation on phospholipase A_2 activity in the sarcolemma of the ischaemic and non-ischaemic regions of the isolated rat heart

Sarcolemma isolated from hearts after 20 minutes coronary artery ligation had a PLA_2 pH dependency similar to that seen with the homogenate, having a pH optimum of 7-8.

Although there was no significant difference between sarcolemma from the ischaemic and non-ischaemic regions there was a tendency towards a decrease in activity in

the ischaemic region (FIG 42).

9.7 Effect of lysophosphatidylcholine and palmitoylcarnitine on phospholipase A_2 activity in homogenates of the isolated rat heart

As inhibition of PLA_2 in the ischaemic region of the isolated rat heart had been shown following 20 minutes ischaemia, a time when the LPC concentration was maximal, the effect of LPC on PLA_2 activity was studied. The effect of PAL was also studied as this is an amphiphile with similar properties to LPC and has also been shown to accumulate during ischaemia (Idell-Wenger et.al. 1978).

LPC produced a stimulation of PLA_2 activity at low concentrations ($\leq$ 50μM) and an inhibition at higher concentrations ($\geq$100μM) (FIG 43).

A similar profile of action on PLA_2 was seen with PAL (FIG 43) with a stimulatory action at $\leq$ 50μM and an inhibitory action at $\geq$200μM.

9.8 Effect of mepacrine on phospholipase A_2 activity in homogenates of the isolated rat heart

Mepacrine, a known inhibitor of PLA_2 (Thakkar, Sperelakis, Pang and Franson 1983), inhibited PLA_2 activity in homogenates of the isolated rat heart at concentrations of 10μM and above (FIG 44). No stimulatory phase was seen, in contrast to the actions of LPC and PAL.

9.9 Effect of lysophosphatidylcholine, palmitoylcarnitine and mepacrine on ADP induced platelet aggregation

LPC and PAL had similar effects on ADP induced platelet aggregation, producing an inhibition at concentrations of 100μM and greater (FIG 45). Mepacrine had a greater inhibitory effect than LPC or PAL on ADP induced platelet aggregation. This paralleled the order of potency of these agents on the inhibition of PLA_2 activity (FIGS 43 + 44).

10 PHOSPHOLIPASE A_2 ACTIVITY IN HEART TISSUE SAMPLES FROM THE ANAESTHETIZED CAT

i) Hearts infused with LPC at final blood concentrations of 20-500μM via the coronary artery

At final blood concentrations of LPC up to 100μM, where no arrhythmias occurred, there was no difference between the PLA_2 activity in the left and right ventricular walls (FIG 46).

Greater LPC concentrations, (300-500μM) which produced arrhythmias and in some cases death from ventricular fibrillation, caused a reduction in PLA_2 activity in the left ventricular wall (FIG 46 + 47). The left ventricular wall would be expected to receive the highest concentrations of LPC and therefore this

inhibition of PLA_2 may be due to direct inhibition by LPC (as shown in FIG 43).

ii) Hearts infused with palmitoylcarnitine at final blood concentrations of 30-75μM via the coronary artery

In hearts infused with PAL at concentrations that produced arrhythmias, and with the higher concentrations death, the PLA_2 activity in the left ventricular wall tended to be depressed (FIG 48 + 49) however this was not significant.

PAL may therefore be causing direct inhibition of PLA_2 (FIG 43) rather than causing changes in the heart which indirectly lead to PLA_2 inhibition.

iii) Hearts with the left anterior descending coronary artery ligated

There was no significant difference between the PLA_2 activity in the ischaemic and non-ischaemic areas of hearts with coronary artery ligation, the activity being 1.50 +/- 0.28 and 1.34 +/- 0.22 x 10^{-11} mol/mg/hr in the ischaemic and non-ischaemic regions respectively (n = 7). This is in contrast to the isolated rat heart. There appeared to be no correlation between severity of coronary artery ligation induced arrhythmias and PLA_2 activity in the ischaemic and non-ischaemic regions.

11 EFFECT OF REPERFUSION OF THE ISCHAEMIC ISOLATED RAT HEART ON PHOSPHOLIPASE A_2 ACTIVITY

11.1 Effect of 10 minutes coronary artery occlusion and 1 minute reperfusion on phospholipase A_2 activity in the isolated rat heart

After 1 minute reperfusion following 10 minutes coronary artery occlusion the PLA_2 activity in the non-ischaemic area was significantly greater than in the ischaemic area, being 7.67 +/- 0.39 and 3.56 +/- 0.40 x 10^{-11} mol/mg/hr respectively (FIG 50). In these experiments a K^+ concentration of 5.9mM was used in the Krebs-Henseleit buffer and no reperfusion arrhythmias were seen. In all the subsequent experiments on reperfusion the K^+ concentration was reduced to 3.2mM as this makes the hearts more susceptible to reperfusion arrhythmias.

11.2 Effect of 10 minutes coronary artery occlusion and 1 minute reperfusion on phospholipase A_2 activity in the isolated rat heart perfused with 3.2mM K^+ Krebs-Henseleit buffer

Using Krebs-Henseleit containing 3.2mM K^+ arrhythmias were seen in all the hearts on reperfusion, in contrast to the 5.9mM K^+ Krebs-Henseleit where no reperfusion arrhythmias were seen.

PLA_2 activity in the non-ischaemic area was

significantly elevated in comparison to that in the ischaemic area (FIG 50), being 9.79 +/- 1.56 and 4.17 +/- 0.70 x 10^{-11} mol/mg/hr respectively. Ten minutes ischaemia only with this composition buffer produced PLA_2 activities of 1.86 +/- 0.07 and 2.21 +/- 0.48 x 10^{-11} mol/mg/hr in the ischaemic and non-ischaemic areas respectively (see section 11.3). Thus reperfusion causes an increase in PLA_2 activity in the non-ischaemic region, regardless of whether arrhythmias occur.

11.3 Effect of 10 minutes coronary artery ligation on phospholipase A_2 activity in the isolated rat heart perfused with Krebs-Henseleit buffer containing 3.2mM K^+

As the composition of the Krebs-Henseleit buffer was changed for the reperfusion experiments the relevant ischaemic and sham ligated controls were carried out.

The course of ischaemically induced arrhythmias was also changed in the hearts perfused with 3.2mM K^+ Krebs-Henseleit. At a potassium concentration of 5.9 mM arrhythmias typically commence following 12 minutes coronary artery ligation and therefore in the occlusion / reperfusion experiments no ischaemic arrhythmias were seen because the occlusion period was only 10 minutes. With 3.2mM potassium Krebs-Henseleit arrhythmias commence at approximately 8 minutes following coronary artery ligation and therefore ischaemic arrhythmias are seen in

these experiments.

Following 10 minutes coronary artery occlusion with the 3.2mM K^+ Krebs-Henseleit buffer there was no significant difference between the PLA_2 activity of the ischaemic (1.86 +/- 0.07 x 10^{-11} mol/mg/hr) and the non-ischaemic (2.21 +/- 0.48 x 10^{-11} mol/mg/hr) areas (FIG 50).

Sham ligated controls showed no difference between the PLA_2 activity in the areas corresponding to the ischaemic and non-ischaemic areas (FIG 50). Although there was no significant difference in PLA_2 activity between the non-ischaemic areas of sham ligated and 10 minute CAL hearts (3.49 +/- 0.55 vs 2.21 +/- 0.48 x 10^{-11} mol/mg/hr) the 10 minute CAL ischaemic area had a significantly lower PLA_2 activity (1.86 +/- 0.07 x 10^{-11} mol/mg/hr) compared with the sham ligated heart (4.57 +/- 0.52 x 10^{-11} mol/mg/hr), $p \leq 0.01$. This is in agreement with the depression in PLA_2 activity seen in ischaemia (FIG 37) although this latter depression was after 20 minutes coronary artery ligation as opposed to 10 minutes.

11.4 Effects of effluent perfusate from reperfused hearts on phospholipase A_2 activity

As activation of PLA_2 in the non-ischaemic region, and to a lesser extent in the ischaemic region, occurred on reperfusion it is possible that the heart is producing something capable of activating PLA_2. Thus the effect of

the perfusate eluting from the reperfused heart on PLA_2 activity was examined.

i) Effects of perfusate on phospholipase A_2 activity

The addition of 30µl of perfusate eluted from a heart reperfused after 10 minutes coronary artery occlusion to the homogenate of a control heart produced an increase in PLA_2 activity from 1.50 +/- 0.16 to 1.98 +/- 0.20 x 10^{-11}mol/mg/hr (FIG 51). Perfusate from the same heart before reperfusion had no effect on PLA_2 activity (FIG 51). Freshly made Krebs-Henseleit buffer had no effect on PLA_2 activity. The effect of the perfusate was stable to its freezing.

ii) Effect of lipid extract of perfusate from a reperfused heart on phospholipase A_2 activity

A lipid extract of the perfusate was made to investigate whether lipids were mediating the effects of the perfusate on PLA_2 activity.

a) The lipid extract of the perfusate from a reperfused heart was tested for its effect on PLA_2 activity in the homogenate of a control heart. As the extract was resuspended in the same volume as the original perfusate the volumes added contained the same concentration of lipid as the perfusate.

At low concentrations (below 50µl) a stimulation of PLA_2 activity was seen, a stimulation being obtained on

addition of 30µl extract, confirming the results with the perfusate. At higher concentrations of extract (greater than 50µl) an inhibition of PLA_2 activity was seen (FIG 52). This type of effect is similar to that seen with LPC and PAL (FIG 43) therefore the lipid extract was separated by TLC to try to identify the lipids.

b) The lipid extract, when separated by TLC, appeared to be composed almost entirely of free fatty acids which comigrated with the oleic acid standard. A small quantity of lipid material remained near the origin which may be LPC or LPE as lysophospholipids remained in this area. As other lipids also remain near the origin these could not be positively identified.

11.5 Effect of a free radical scavenger on phospholipase A_2 activity during reperfusion

It is possible that free radicals have a role in changes occuring during reperfusion (see section 1.8) and thus a free radical scavenger was tested for its ability to modulate the changes in PLA_2 activity seen on reperfusion.

The presence of 1mM reduced glutathione in the perfusate reduced the PLA_2 activity seen on reperfusion following 10 minutes coronary artery ligation. This is in comparison to the identical experiment without glutathione (FIG 53), with PLA_2 activity being 9.79 +/- 1.56 and 4.62 +/- 0.41 x 10^{-11} mol/mg/hr without and with

glutathione respectively.

The activity in the non-ischaemic area (4.62 +/- 0.41 x 10^{-11} mol/mg/hr) is still significantly greater than in the ischaemic area (2.63 +/- 0.27 x 10^{-11} mol/mg/hr, $p \leq 0.02$) and significantly greater than in the non-ischaemic area after 10 minutes ischaemia (2.21 +/- 0.48 x 10^{-11} mol/mg/hr).

11.6 Effect of a constant pressure head system on phospholipase A_2 activity during reperfusion of the isolated rat heart

During coronary artery occlusion there would be expected to be an increase in flow in the non-ischaemic region using a constant flow system. As these flow changes may cause changes in PLA_2 activity independently of ischaemia and reperfusion a constant pressure head system was used to reduce any flow changes.

PLA_2 activity after 1 minute reperfusion following 10 minutes occlusion was the same in the ischaemic and non-ischaemic regions (3.76 +/- 0.42 vs 3.9 +/- 1.32 x 10^{-11} mol/mg/hr) (FIG 54). This represented a significant decrease in activity in the non-ischaemic region between the constant flow (9.79 +/- 1.56 x 10^{-11} mol/mg/hr) and the constant pressure systems (3.9 +/- 1.32 x 10^{-11} mol/mg/hr) (FIG 54).

11.7 Effect of flow rate changes on phospholipase A_2 activity in the isolated rat heart

As ischaemia and reperfusion in a constant flow system produced differences in PLA_2 activity from ischaemia and reperfusion in a constant pressure head system the effect of flow changes on PLA_2 activity were investigated.

a) Hearts perfused at 15ml/min for 10 minutes had a higher PLA_2 activity (8.41 +/- 1.33 x 10^{-11} mol/mg/hr) than hearts subsequently perfused at 10ml/min for 1 minute (5.2 +/- 1.16 x 10^{-11} mol/mg/hr) although this difference was not significant (FIG 55). These two regimes simulate the flow changes seen in the non-ischaemic region of the CAL, and CAL plus reperfused hearts respectively.

b) Hearts made globally ischaemic for 10 minutes had a significantly higher PLA_2 activity than hearts subsequently perfused at 10ml/min for 1 minute (5.50 +/- 0.69 vs 2.83 +/- 0.25 x 10^{-11} mol/mg/hr) (FIG 55). Although the hearts were perfused in a heated water jacket the temperature of the heart decreased during global ischaemia. This may cause changes in PLA_2 activity which are unrelated to the flow changes but are a function of the decreased temperature. The temperature in the ischaemic area of the coronary artery ligated heart would be expected to fall but not to the same extent as

during global ischaemia.

Therefore changes in the flow rate of the whole heart are able to produce changes in PLA_2 activity independently of any effects of ischaemia and reperfusion, increased flow producing increased PLA_2 activity.

11.8 Effect of vasodilators on phospholipase A_2 activity in the isolated rat heart

As increased flow produced an increase in PLA_2 activity it is possible that increased shear and stress forces in the vasculature lead to damage and raised PLA_2 activity. Thus the effects of two vasodilators were investigated to see if a reduction in stress affected PLA_2 activity.

a) Effect of adenosine on phospholipase A_2 activity during high flow

A concentration of 10^{-5}M adenosine was chosen as it has been reported to cause maximal vasodilation (Schrader, Haddy and Gerlach 1977) and during preliminary experiments a maximal effect was seen on the perfusion pressure reduction in an isolated rat heart with this concentration of adenosine contained in Krebs-Henseleit buffer.

Significant differences were seen between the effect of adenosine in recirculating and non-recirculating

systems.

A 10 minute perfusion with 10^{-5}M adenosine in a recirculating system produced no effect on the elevated PLA_2 activity seen on perfusing a heart at 15ml/min instead of 10ml/min (9.85 +/- 1.29 x 10^{-11} mol/mg/hr with adenosine in comparison to 8.41 +/- 1.33 x10^{-11} mol/mg/hr without adenosine). As it is possible that adenosine is metabolised by the tissue in the 5 minute perfusion prior to elevation of flow rate from 10 to 15 ml/min the same experiment was carried out with a non-recirculating system where this metabolism should not occur.

In a non-recirculating system 10^{-5}M adenosine produced a significant decrease in PLA_2 activity (3.7 +/- 0.55 x 10^{-11} mol/mg/hr from 8.41 +/- 1.33 x 10^{-11} mol/mg/hr) while 10^{-6}M adenosine had an intermediate effect (FIG 56).

As there is a significant decrease between the effect of 10^{-5}M adenosine on PLA_2 activity in a recirculating and a non-recirculating system the assumption that metabolism of the adenosine by the tissue occurs is probably correct.

b) Effect of sodium nitroprusside on phospholipase A_2 activity during high flow

In preliminary experiments 10^{-4}M sodium nitroprusside was shown to produce maximal vasodilation in the whole rat heart in a recirculating system.

Hearts perfused at a flow rate of 15ml/min (high flow rate) for 10 minutes with 10^{-4}M sodium nitroprusside containing Krebs-Henseleit buffer exhibited a lower PLA_2 activity (4.12 +/- 0.16 x 10^{-11} mol/mg/hr) than those perfused without sodium nitroprusside (8.14 +/- 1.33 x 10^{-11} mol/mg/hr) (FIG 57).

Thus both vasodilators adenosine and sodium nitroprusside are capable of antagonising the effect of a high flow rate on PLA_2 activity in the whole heart. Therefore the increase in PLA_2 activity at high flow rate may be due to stress forces within the vasculature.

9 DISCUSSION

PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART

Characterisation of phospholipase A_2 activity in the homogenate of the isolated rat heart

The release of C^{14}-oleic acid from PE labelled with the oleic acid in the 2-acyl position was shown to be proportional to the protein concentration and to be totally attributable to enzymic activity when compared to controls run in parallel with no protein present (FIG 32). This release of C^{14}-oleic acid was calcium dependent, reaching a maximum at 3-5mM Ca^{2+} (FIG 33),and was linear with time until at least 45 minutes (FIG 34). This calcium dependency is similar to that of phospholipase A_2 activity in rat aorta smooth muscle (Thakkar et.al. 1983), rat and human lymphocytes (Etienne and Polonovski 1984), rat platelets (Withnall et.al. 1984), and subcellular fractions of the rat heart (Nalbone and Hostetler 1985). In common with the work of Nalbone and Hostetler (1985) EDTA did not produce complete inhibition of the activity, suggesting the presence of a calcium independent enzyme. A plasmalogen specific calcium independent PLA_2 has been reported in canine myocardium which has low activity for diacylphospholipids (Wolf and Gross 1985) therefore this could account for the activity seen in the presence of EDTA. The Km for calcium was calculated to be 0.6mM by

the direct linear plot method, a value similar to that reported for platelet PLA_2 (Watanabe et.al.1986).

The maximal activity seen was approximately 2.5 x 10^{-11} mol/mg/hr. This is 100 times lower than many of the reported values in the literature (Weglicki et.al. 1971, Franson et.al. 1978, Corr, Ahumada and Sobel 1982, Nalbone and Hostetler 1985). This may be due to the different modes of substrate preparation. In most cases labelled substrate of known specific activity is diluted with an identical unlabelled substrate. However, when labelled substrate is prepared using rat liver microsomes, as in this thesis, the labelled lipid will also be diluted with other phospholipids present in these microsomal membranes as they will all be extracted and isolated together with the labelled substrate. It was not thought valid to measure the total phospholipid concentration in the substrate aliquots and assume that this is the effective substrate concentration as under most conditions PC and PE form entirely different types of aggregates (Waite 1985) and PE is a better substrate for PLA_2 than PC (Weglicki et.al. 1971, Wurl and Kunze 1985). However as PLA_2 is capable of acting on other phospholipids apart from PE the total concentration of the latter is also not an accurate reflection of the substrate concentration. The activity was thus calculated for the labelled substrate only. In each assay labelled PE of 40,000 dpm was used. At a specific activity of

57mCi/mmol of C^{14}-oleic acid this is equivalent to 2.1μM of labelled PE.

The rat liver microsomes used in the preparation of the labelled substrate had a protein concentration of 10mg/ml. As 11ml of microsomes were used , lipid to protein content is approximately 2μmol/mg protein in lipid bilayers (Philipson, Bers and Nishimoto 1980) and 120ml of substrate solution was prepared there would be 1.32×10^{-4} moles in 120ml of substrate. Thus in 20μl there would be 2.20×10^{-8} mols of lipid. This in the assay would give an unlabelled lipid concentration of 146μM. Therefore if PLA_2 acts equally on all the phospholipids the activity is 73 times greater than quoted, i.e approximately 2nmol/mg/hr. This is in the range of activities quoted by other workers. The proportion of hydrolysed labelled substrate did not exceed 4%, in common with the results of Ballou and Cheung (1985) with platelet PLA_2 and thus it is likely that unlabelled and labelled substrates are degraded equally. The PLA_2 activity , as measured against exogenously added substrate, does not necessarily reflect that towards endogenous substrates in the *in vivo* situation where enzyme and substrate are in the same membrane. Kinetic parameters for mitochondrial PLA_2 against endogenous and exogenous substrates have been shown to differ (De Winter, Korpancova and van den Bosch 1984). The saturating substrate concentration used in this thesis

allowed assessment of activity towards exogenous substrate. Although it would be possible to determine kinetic constants for the exogenous substrate there is little meaning in such values as solution kinetics do not apply when the substrate is above its critical micelle concentration (Waite 1985).

SUMMARY: PLA_2 activity in the rat heart homogenate was shown to be proportional to protein concentration, time and calcium dependent with a Km of approximately 0.6mM Ca^{2+}.

Effect of coronary artery ligation on phospholipase A_2 activity

PLA_2 in the homogenate of the isolated rat heart showed a pH optimum of 7-8 in both ischaemic and non-ischaemic regions. This alkaline pH optimum is consistent with that found for membrane bound PLA_2 (van den Bosch 1980, Etienne and Polonovski 1984). Some activity was seen at acidic pH 4-5. This may be due to the activity of a lysosomal soluble PLA_2 which is active at this pH, however the majority of phospholipase activity at pH 4 has been reported to be of the A_1 type rather than A_2 (Nalbone and Hostetler 1985) therefore this may explain why very little activity was seen at acidic pH.

Following 5 minutes coronary artery ligation there was no difference between PLA_2 activity in the ischaemic

and non-ischaemic areas of the isolated rat heart (FIG 36). However following 20 minutes coronary artery ligation the activity in the ischaemic region was reduced (FIG 37). Thus at a time when the lysophospholipid concentration and severity of arrhythmias are at their peak the activity of PLA_2 is actually inhibited. This does not support the hypothesis that PLA_2 activity increases during ischaemia and is responsible for the increased lysophospholipid concentrations. Although the enzyme is calcium dependent any regulation of PLA_2 by calcium will not be seen in this assay as it is carried out at a calcium concentration producing maximal activity. It is possible that an initial increase in intracellular free calcium could cause PLA_2 activation before the inhibition of PLA_2 at 20 minutes ischaemia however evidence for increased free calcium concentrations during the early phase of ischaemia is lacking. In rabbit septal preparations cytosolic calcium is reported to be less than 100μM during ischaemia and in the Langendorff perfused rabbit heart a free calcium concentration of only 40μM would be obtained if intracellular calcium was homogenously distributed (Poole-Wilson et.al. 1984). Similarly it has been suggested that free calcium during ischaemia does not exceed 25μM (Jennings et.al. 1985). These values are considerably lower than the quoted Km for calcium, casting doubt on the ability of calcium to exert a

regulatory role during ischaemia.

Inhibition of PLA_2 following periods of ischaemia greater than five minutes has been reported in the gerbil brain (Edgar et.al. 1982) and was attributed to inhibition by free fatty acids. When the effect of LPC, a product of PLA_2 action, on PLA_2 activity was studied (FIG 43) it was seen that after an initial stimulation at low concentrations, an inhibition of activity was seen at concentrations of LPC above 100μM. This profile of activity was similar to that of another amphiphile, oleoyl CoA, on Na^+ / K^+ ATPase (Weglicki, Kramer, Franson, Pang and Owens 1985). The LPC concentration in the rat heart following 20 minutes coronary artery ligation was previously calculated to be 1.2mM. As a dilution of the tissue of 18 occurs in the assay (6 in homogenisation and a further 3 in the assay itself) the LPC concentration in the PLA_2 assay of the ischaemic area following 20 minutes coronary artery ligation is likely to be approx. 70μM. This does not appear sufficient to cause inhibition of PLA_2 however this calculation assumes homogenous distribution of LPC within the tissue. As LPC readily incorporates into membranes the local concentration of LPC near the PLA_2 in the membrane is likely to be much higher. During ischaemia LPC is produced by PLA_2 action and is therefore already situated within the membrane bilayer in close proximity to the enzyme. It is thus possible that inhibition will occur.

PAL was also shown to have a similar inhibitory action on PLA_2 (FIG 43) and concentrations of PAL reach millimolar levels during ischaemia (Idell-Wenger et.al. 1978, Shug, Thornsen, Folts, Bittar, Klein, Koke and Huth 1978). PAL has been shown to accumulate primarily in the sarcolemma during hypoxia (Knabb et.al. 1986) although other workers found accumulation was largely in the cytosol during ischaemia (Idell-Wenger et.al. 1978) and this could also contribute to inhibition of PLA_2 during ischaemia.

Synergy has been reported in the inhibitory action of PAL and other amphiphiles , such that two non-inhibitory concentrations would produce an effect when added together , on the activity of myocyte sarcolemmal Na^+/K^+ ATPase (Weglicki et.al. 1985) and therefore it is possible that this may occur in the heart with LPC and PAL.

SUMMARY: PLA_2 activity in the rat heart homogenate was shown to be the same in the ischaemic and non-ischaemic regions following 5 minutes coronary artery ligation however at 20 minutes post ligation the activity in the ischaemic region was reduced. This may be due to inhibition by LPC and PAL, both of which were shown to inhibit PLA_2 when added exogenously.

Mitochondrial phospholipase A_2

Mitochondrial PLA_2 activity showed a broad pH optimum at neutral pH and calcium dependency (FIGS 38 +39). Maximal activity was seen at a calcium concentration of 3-5mM. Some activity was seen in the presence of 10mM EDTA. This could be due to the presence of a calcium independent enzyme or calcium binding to the mitochondria. It has been reported that washing of mitochondria in sucrose/EDTA does not remove all the calcium from the mitochondria (Bjørnstad 1966).

Following 20 minutes coronary artery ligation, at a time when the activity of PLA_2 in a nomogenate of the ischaemic region of the isolated rat heart is depressed, there also appeared to be a reduced mitochondrial PLA_2 activity in the ischaemic region (FIG 40). This inhibition may be due to LPC or PAL accumulation within the membranes. Isolation of mitochondria from ischaemic tissue may result in the selective isolation of the least damaged organelles as it has been shown that isolation of canine cardiac sarcoplasmic reticulum is selective during ischaemia, in this case for the more damaged organelles (Rapundalo, Briggs and Feher 1986). Slight mitochondrial damage has been seen following 15-20 minutes ischaemia (Schaper 1979) thus it is possible that the functionally more normal mitochondria have been isolated . Two different mitochondrial populations also appear to exist, depending on the mode of preparation (Nalbone and

Hostetler 1985) and therefore these may be affected differently by ischaemia.

Sarcolemmal phospholipase A_2

The calcium dependency of sarcolemmal PLA_2 was similar to that of the mitochondria and the whole heart homogenate with maximal activity at 3-5mM calcium (FIG 41). With this preparation there was still substantial activity in the presence of 10mM EDTA. Again this may represent a calcium independent activity. As the sarcolemma will be present as vesicles it is possible that these will retain calcium from the initial homogenisation and this would not be accessible to EDTA. Sarcolemmal phospholipids such as PS and PI are also known to have an important role in calcium binding (Philipson et.al. 1980, Langer 1985) as have the glycocalyx sialic acids (Langer, Frank, Nudd and Seraydarian 1976, Nathan and Bhattachayya 1981) and this bound calcium may be retained through the isolation procedure.

The pH profile of sarcolemmal PLA_2, in common with that of the homogenate and mitochondria, showed a pH optimum at pH 7-8 (FIG 42). There appeared to be no difference in the PLA_2 activity in the sarcolemma between the ischaemic and non-ischaemic areas following 20 minutes coronary artery ligation (FIG 42). This could indicate that the PLA_2 activity is unchanged during

ischaemia , the change seen in the homogenate being due to that in the mitochondria, or it may be a result of removal of inhibitory moieties during the isolation procedure of the sarcolemma.

SUMMARY: Mitochondrial and sarcolemmal PLA_2 both showed pH and calcium dependencies similar to that of the homogenate. During ischaemia depression of activity in the ischaemic region was seen in the mitochondria but not in the sarcolemma which may be due to differences in the enzyme or washout of inhibitory substances during the isolation of the sarcolemma.

Effect of lysophosphatidylcholine, palmitoylcarnitine and mepacrine on phospholipase A_2 activity

LPC and PAL both showed a similar profile of effect on PLA_2 activity, an initial stimulation at low concentrations followed by an inhibition at greater concentrations (FIG 43). As already discussed this could be a causative factor in the inhibition of PLA_2 during ischaemia.

Stimulation seen at low amphiphile concentrations could be due to unmasking of latent PLA_2 activity that was not previously accessible to the substrate. Changes in membrane dynamics and fluidity produced by amphiphiles (Fink and Gross 1984) may allow accessibility of PLA_2 that is shielded from the substrate. The inhibition of

PLA_2 by LPC may be by product inhibition as suggested by Lawrence (1975) but as the same effect is produced by PAL it is more likely to be a non-specific inhibition. It has previously been suggested that 2-acyl lysophospholipids are weak inhibitors of PLA_2 (Wolf and Gross 1984) and drugs with diverse amphiphilic structures such as chlorpromazine and imipramine have also been reported to inhibit PLA_2 (Hostetler and Matzuzuawa 1981). The latter compounds have been shown to have a biphasic profile of action on isolated PLA_2 (Kunze, Nahas, Traynor and Wurl 1976), however the effect was dependent on substrate presentation and enzyme source. These types of interaction did not appear to be due to substrate competition.

Mepacrine also inhibited PLA_2 activity of the isolated rat heart, however no stimulatory phase was seen at low concentrations unlike LPC and PAL (FIG 44). The mechanism of its inhibition is thus likely to be different from that of LPC and PAL. Inhibition by mepacrine was seen at lower concentrations than reported elsewhere (Flower and Blackwell 1976, Thakkar et. al. 1983, Glaser and Jacobs 1986) however as these reports refer to different systems it is difficult to draw a true comparison as mepacrine is thought to act non-specifically by binding to membrane phospholipid (Dise, Burch and Goodman 1982).

The effect of added LPC on rat heart PLA_2 (FIG 45)

was qualitatively similar to that seen on the calcium / ATPase of rabbit skeletal muscle sarcoplasmic reticulum (Martonosi, Donley and Halpin 1968, Martonosi, Donley, Purcell and Halpin 1971). In the latter case increased Ca^{2+} / ATPase activity was seen at LPC concentrations less than 150μM whilst inhibition was seen at concentrations greater than this. However when the same membranes were treated with PLA_2 to increase the production of LPC and fatty acids endogenously no stimulatory phase was seen on addition of exogenous LPC. It is therefore possible that in vivo stimulation of PLA_2 by low concentrations of LPC will not be seen.

As both PLA_2 and phospholipase C are thought to be involved in the aggregatory response of platelets to ADP (Bills, Smith and Silver 1976, Bell et.al. 1979, Simon, Chap and Douste-Blazy 1986) the effect of LPC, PAL and mepacrine on ADP induced platelet aggregation was investigated. Both LPC and PAL had an inhibitory effect on aggregation at concentrations greater than 50μM, an effect similar to their action on PLA_2. Mepacrine had a much stronger inhibitory effect than LPC and PAL which may reflect inhibition of PLC by mepacrine in addition to PLA_2, mepacrine and similar compounds having been shown to inhibit macrophage PLC (Wightman, Dahlgren, Hall, Davies and Bonney 1981) and human platelet PI-specific PLC (Hofmann, Prescott and Majerus 1982). It is not known whether LPC or PAL inhibit PLC and thus if this is the

case the stronger inhibitory effect of mepacrine on ADP induced platelet aggregation may be due to another cause. The majority of the PLC activities studied have been reported to be of cytosolic origin (Waite 1985, Wolf and Gross 1985). The interactions of LPC and PAL with PLA_2 and PLC may differ as the former is membrane bound and the amphiphiles may act by intercalation into the membrane. The correlation between platelet aggregation and release (by PLA_2 or PLC) and metabolism of arachidonic acid is not absolute as α-tocopherol has been shown to be anti-aggregatory but had no effect on the concentrations of arachidonic acid metabolites in platelets (Srivastava 1986) although other workers have found an inhibitory effect of α-tocopherol on platelet PLA_2 (Douglas, Chan and Choy 1986).

SUMMARY: LPC and PAL are capable of inhibiting PLA_2, therefore increased concentrations of these two compounds during ischaemia may explain the inhibition of PLA_2. LPC and PAL both inhibit ADP induced platelet aggregation as does mepacrine, possibly due to inhibition of phospholipases. Therefore the production of ischaemia by PAL in the anaesthetized cat is unlikely to be due to a direct effect on platelet aggregation or the potentiation of aggregation induced by another factor.

10 PHOSPHOLIPASE A_2 ACTIVITY IN HEART TISSUE SAMPLES FROM THE ANAESTHETIZED CAT

In hearts infused with concentrations of LPC sufficient to produce arrhythmias PLA_2 activity was depressed in the tissue perfused by the left anterior descending coronary artery (FIGS 46 + 47). This implies that during ischaemia, when LPC concentrations are elevated sufficiently to cause arrhythmias, this concentration may be sufficient to cause suppression of PLA_2 activity. It is therefore unlikely that there would be a maintained increase in PLA_2 activity during ischaemia.

There does not appear to be as great an effect on PLA_2 activity in tissue from hearts infused with arrhythmogenic concentrations of PAL (FIGS 48 + 49). This is perhaps to be expected as lower concentrations of PAL than LPC produced arrhythmias thus it is likely that there is less PAL present within tne cells.

Unlike the isolated rat heart there was no difference in PLA_2 activity in the ischaemic and non-ischaemic areas of the cat heart following coronary artery ligation. During ischaemia other factors are also thought to be involved with the production of arrhythmias. The reduced pH in ischaemia increases the susceptibility of Purkinje fibres to LPC induced electrophysiological changes (Corr and Snyder et.al.

1981), thus arrhythmias may be produced in the ischaemic heart at lower LPC concentrations than in the normoxic heart by LPC infusion. However these factors should also apply in the isolated rat heart unless collateral flow is substantially greater in the cat, allowing washout of LPC from the ischaemic area. Considerable collateral flow was seen in the cat heart in some experiments as coronary artery ligation occasionally did not produce either a well defined ischaemic area, as visualised by dye (i.v.), or a fall in temperature in the ischaemic area. These were a separate set of experiments. The presence of blood in the *in vivo* cat heart is also likely to facilitate washout of LPC due to binding to albumin.

The PLA_2 activities measured in the cat heart were considerably lower than in the isolated rat heart. This is in agreement with differences seen between *in vivo* and *in vitro* rabbit hearts (review Corr, Ahumada and Sobel 1981). Some loss of activity occurred due to storage at -20°C for prolonged periods of time (section 9.1 iv) and it is also possible that there are inhibitory factors in the blood trapped within the tissue (Miwa, Ichihashi, Motojima, Onodera-Kubota, Matsumoto 1985). Species difference can also not be excluded. Thus although it is valid to compare results between the different cat heart samples comparison with the rat heart has little meaning.

SUMMARY: LPC, at concentrations causing arrhythmias when infused into the left descending coronary artery of the anaesthetized cat, caused a depression of PLA_2 activity in the left ventricular wall. PAL, at arrhythmogenic concentrations , produced a smaller non significant depression of PLA_2 activity, possibly due to the smaller concentrations of PAL needed to produce arrhythmias. Ischaemia did not produce inhibition of PLA_2. This may be due to the large degree of collateral flow in the cat heart allowing washout of the LPC and PAL produced during ischaemia. Activities were lower than in the isolated rat heart, probably due to loss of activity during freezing and the effects of the presence of blood in the *in vivo* cat heart.

11 EFFECT OF REPERFUSION OF THE ISCHAEMIC ISOLATED RAT HEART ON PHOSPHOLIPASE A_2 ACTIVITY

Effect of reperfusion and free radical scavengers on phospholipase A_2 activity

On reperfusion of the isolated rat heart following 10 minutes coronary artery occlusion PLA_2 activity was significantly greater in the non-ischaemic region than in the ischaemic region (FIG 50). This occurred with perfusates containing either 3.2mM or 5.9mM K^+, the former predisposing the hearts to reperfusion arrhythmias, none occuring with 5.9mM K^+. The difference

in activity between the ischaemic and non-ischaemic regions appeared to be due to an increase in activity in the non-ischaemic area rather than a decrease in activity in the ischaemic region (as seen during ischaemia).

PLA_2 activity in the ischaemic region of hearts ligated for 10 minutes was reduced in comparison with that in sham ligated hearts, again confirming a depression of PLA_2 activity during ischaemia (FIG 50). Although there appeared to be a slightly elevated PLA_2 activity in the ischaemic region during reperfusion this was not significant.

During reperfusion of the ischaemic tissue it is possible that something capable of activating PLA_2 is produced or some inhibitory substance already present is washed out leading to activation in the non-ischaemic region. When the effect of perfusate from ischaemic and reperfused hearts on control PLA_2 activity was determined there appeared to be something present only in the latter that could increase PLA_2 activity (FIG 51). The concentration dependent effect of this perfusate was qualitatively similar to that of LPC and PAL on PLA_2. As shown by TLC this may be due to fatty acids or LPC in the perfusate and thus the change seen in PLA_2 activity may be due to washout from the ischaemic area. However, if this is the case it would have been expected that activity would also have been elevated in the ischaemic area unless it is a function of concentration.

Alternatively changes in the ischaemic area during reperfusion lead to the production of something able to activate PLA_2 in the non-ischaemic region. Reduced glutathione, a scavenger of hydrogen peroxide (FIG 10), produced a reduction in the PLA_2 activity in the non-ischaemic region following reperfusion although the activity was still greater than that in the ischaemic region (FIG 53). This may indicate that free radical mechanisms do have a role in the increase in PLA_2 activity seen on reperfusion however glutathione also acts as a vasodilator, in common with other free radical scavengers (Lucas, Gardner, Flaherty, Bulkley, Elmer and Gott 1980) and this may have a separate effect on PLA_2 activity.

During reperfusion there is rapid, severe damage to the myocardial cells (Hearse et.al. 1973, Hearse, Humphrey, Naylor, Slade and Border 1975). A major cause of this reperfusion injury is thought to be the production of free radicals (FIG 10), as demonstrated by the protective effects of free radical scavengers such as reduced glutathione, catalase, superoxide dismutase and mannitol (Lucas et. al. 1980, Shlafer, Kane and Kirsch 1982, Stewart, Blackwell, Crute, Loughlin, Greenfield and Hess 1983). Free radical formation is thought to contribute to myocardial membrane damage by initiating a chain reaction of lipid peroxidation (Svingen, Buege, O'Neal and Aust 1979, review Meerson, Kagan, Kozlov,

Belkina and Arkipenko 1982). During the propagation reactions a number of products are formed (Bernheim, Bernheim and Wilbur 1948), one of which (malondialdehyde) is often assayed to provide a measure of lipid peroxidation (Bird and Draper 1984).

As free radicals lead to the degradation of membrane polyunsaturated fatty acids the membrane structure is disorganised and can lead to disturbance of its function due to changes in the lipid environment of membrane bound enzymes and formation of new permeability channels (Goldstein and Weissman 1977).

It is thus possible that lipid peroxidation products could wash out of the ischaemic region on reperfusion and cause perturbation of the membrane structure in the non-ischaemic region and lead to activation of PLA_2. It is unlikely to be a direct effect of free radicals as they have a very short half life and will react before reaching the non-ischaemic region. Lipid amphiphiles such as LPC and PAL, that would wash out of the ischaemic region upon reperfusion, have been shown to potentiate free radical induced lipid peroxidative injury to sarcolemmal membranes (Mak et.al. 1986) although other workers reported an inhibition of lipid peroxidation *in vitro* by LPC (Kihlstrom and Saiminen 1985) and thus LPC and PAL may alter the effects of free radicals.

Effect of flow changes and vasodilators on phospholipase A_2 activity

As the standard perfusion uses a constant flow (10ml/min), ischaemia produced by coronary artery ligation would be expected to lead to an increase in flow in the non-ischaemic region which would then decrease on reperfusion. It is possible that the changes in flow may contribute to the changes in PLA_2. Using a constant pressure head system no such changes in flow would be expected to occur and therefore the effects of flow changes could be investigated separately from those of reperfusion. With a constant pressure system there was no increase in PLA_2 activity upon reperfusion and therefore it is possible that changes in flow are contributing to changes in PLA_2 activity under the constant flow system (FIG 54).

To examine further the effects of flow rate changes on PLA_2 its activity was determined in whole hearts subjected to flow changes simulating those of ischaemia and reperfusion in both the ischaemic and non-ischaemic regions. Increasing flow (FIG 55 A + C) increased PLA_2 activity. The activity seen on increased flow (FIG 55 C) is much greater than that seen in the non-ischaemic region of a heart subjected to coronary artery ligation (FIG 40). This may reflect the lack, in the former, of inhibition by recirculation of substances such as LPC, that have washed out of the ischaemic area.

Activity in FIG 55 (A), where increased flow was followed by a return to normal flow, simulating the changes seen in the non-ischaemic region of the reperfused heart, was less than that seen in the reperfused heart. This may be due to a contributory role of free radical damage which would apply on reperfusion.

The previously ischaemic region of the reperfused heart showed no significant difference in PLA_2 activity from FIG 55 (B) i.e. from its simulated flow change in the whole heart. However there was a greater activity when the flow change of ischaemia, i.e. zero flow, was simulated than in the ischaemic area of a coronary artery ligated heart (5.495 +/- 0.69 vs 1.863 +/- 0.066 x 10^{-11} mol/mg/hr). This may be a function of different metabolic changes occuring in the globally, rather than regionally, ischaemic heart especially as the temperature of the former was significantly reduced , and thus enzyme activity leading to the production of possibly inhibitory moieties will be depressed.

Flow changes may cause endothelial damage. Functional and morphological abnormalities of the brain vasculature (both endothelial and vasculature smooth muscle) have been seen following an elevation of blood pressure (Kontos, Wei, Dietrich, Navari, Povlishock, Ghatak, Ellis and Patterson 1981). It has been suggested that this causes phospholipase activation in the vessel wall (Ellis, Wright, Wei and Kontos 1981, Wei, Lamb and

Kontos 1982) although the mechanism of activation is unknown. The products of arachidonic acid metabolism appeared to mediate the vascular injury seen.

The effects of vasodilators on the increased PLA_2 activity induced by increased flow was studied to determine whether reduced vascular resistance may limit any damage and thus reduce PLA_2 activity. A concentration of adenosine that produces maximal vasodilation ($10^{-5}M$) in a non-recirculating system reduced the PLA_2 activity seen on increasing flow to 15ml/min (FIG 56), as did $10^{-4}M$ sodium nitroprusside (FIG 57). It is thus possible that vasodilators reduce the shear stress caused by high flow rates and reduce vascular damage and PLA_2 activation.

It cannot be excluded that the vasodilators are acting by another mechanism. Sodium nitroprusside is thought to act by increasing cGMP content (Gruetter, Gruetter, Lyon, Kadowitz and Ignarro 1981) and adenosine may act by interfering with calcium utilisation (review Berne 1980). It could therefore be other effects of these cGMP and calcium systems on PLA_2 rather than the resultant vasodilation that leads to changes in PLA_2 activity.

SUMMARY: Reperfusion causes slight increases in PLA_2 activity in the ischaemic area and significant increases in the non-ischaemic area. This is likely to be partly

due to flow changes as some of the changes in activity could be simulated by changes in flow rates in whole hearts. Free radicals may also have a role as no increase in PLA_2 activity was seen in the non-ischaemic area of a coronary artery ligated heart where the same flow changes would be expected to occur as when flow was increased in a whole heart. Reduced glutathione, however, was able to reduce the activity of PLA_2 seen in the non-ischaemic area on reperfusion. Free radicals appear to have a role in the injury caused by vascular damage (Wei, Kontos, Dietrich, Povlishock and Ellis 1981) thus any damage produced by high flow may be potentiated by free radicals from the ischaemic area and lead to PLA_2 activation by changes in membrane structure and fluidity.

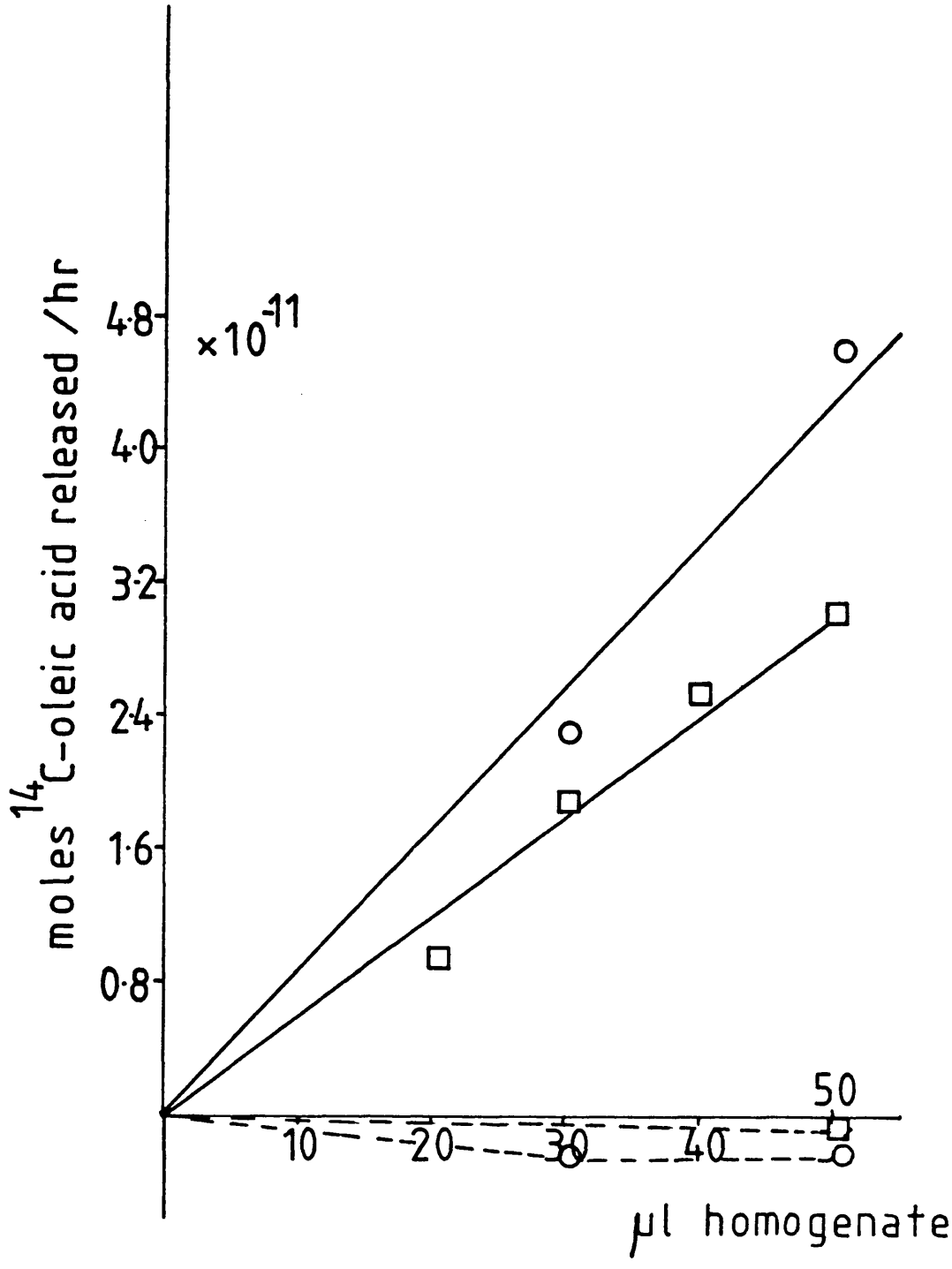


FIG 32. PROTEIN DEPENDENCE OF PHOSPHOLIPASE A_2 ASSAY IN THE HOMOGENATE OF THE ISOLATED RAT HEART. ---BOILED HOMOGENATE.

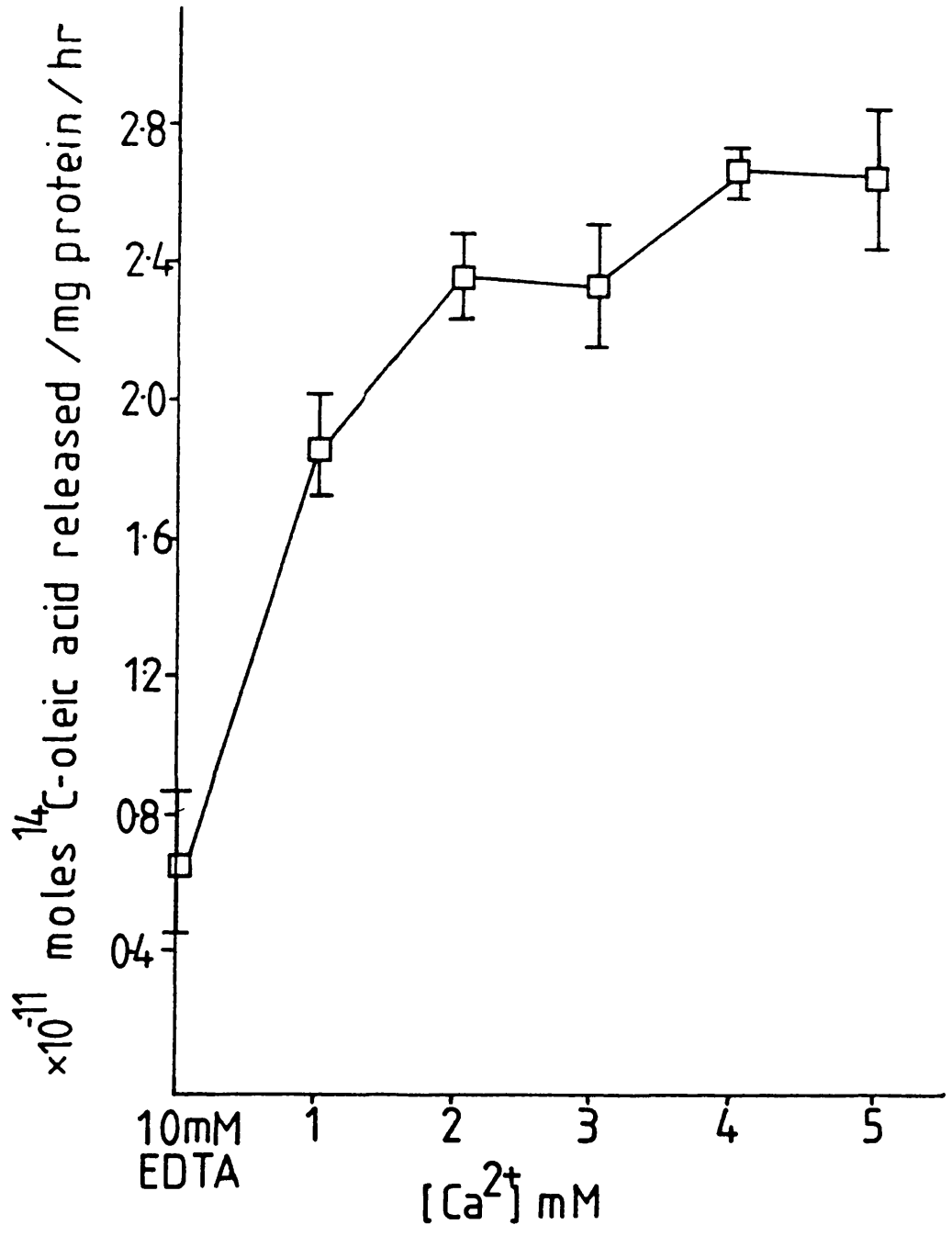


FIG 33. CALCIUM DEPENDENCE OF PHOSPHOLIPASE A_2 ACTIVITY IN THE HOMOGENATE OF THE ISOLATED RAT HEART. MEAN $\pm$ SEM.n = 4.

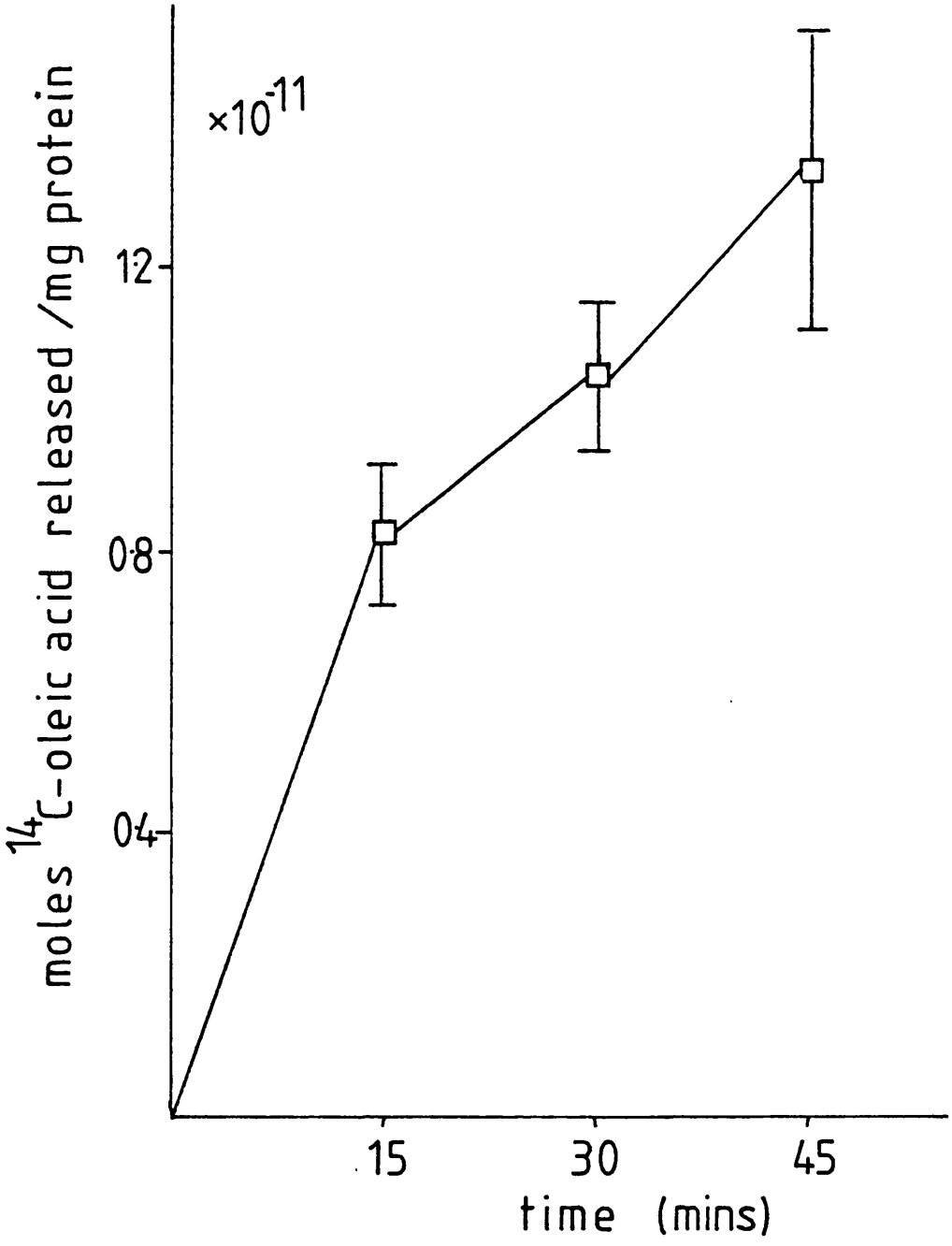


FIG 34. TIME DEPENDENCY OF PHOSPHOLIPASE A_2 ASSAY IN A HOMOGENATE OF THE ISOLATED RAT HEART. MEAN ± SEM. n = 2.

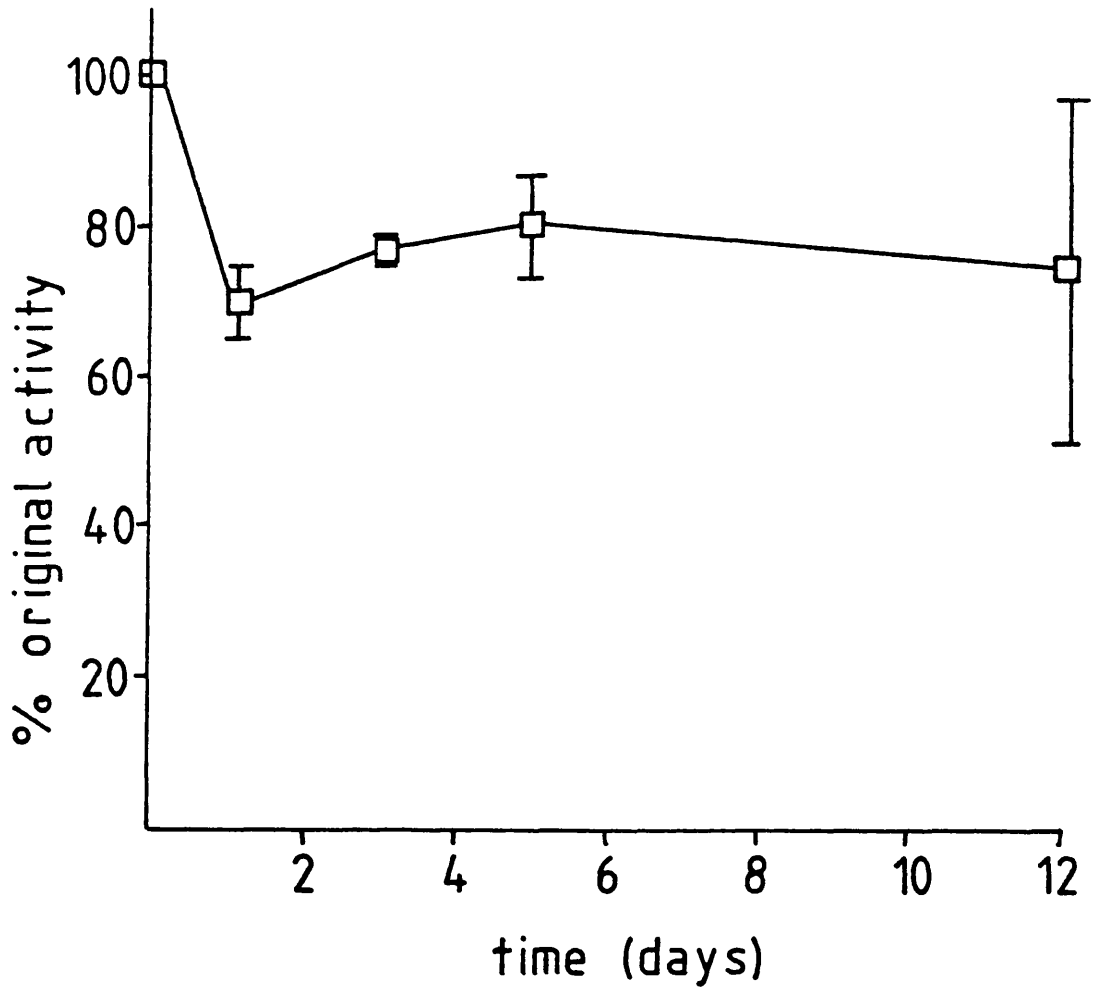


FIG 35. TIME COURSE OF LOSS OF PHOSPHOLIPASE A_2 ACTIVITY FOLLOWING FREEZING. MEAN $\pm$ SEM. n = 3.

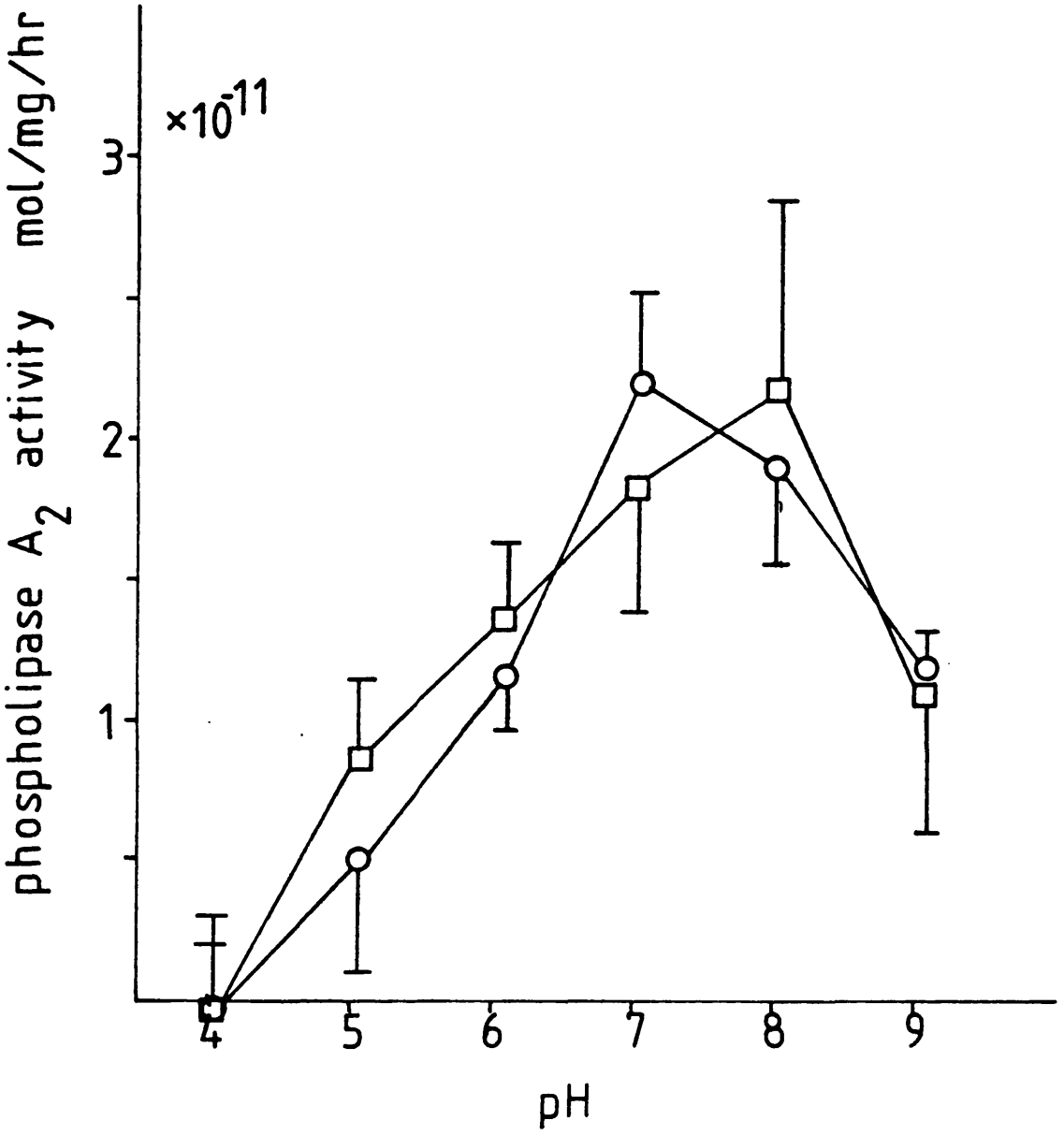


FIG 36. pH PROFILE OF PHOSPHOLIPASE A_2 ACTIVITY IN THE HOMOGENATE OF THE ISOLATED RAT HEART FOLLOWING 5 MINUTES CORONARY ARTERY LIGATION. MEAN $\pm$ SEM. n = 3. □ NON-ISCHAEMIC AREA. ○ ISCHAEMIC AREA.

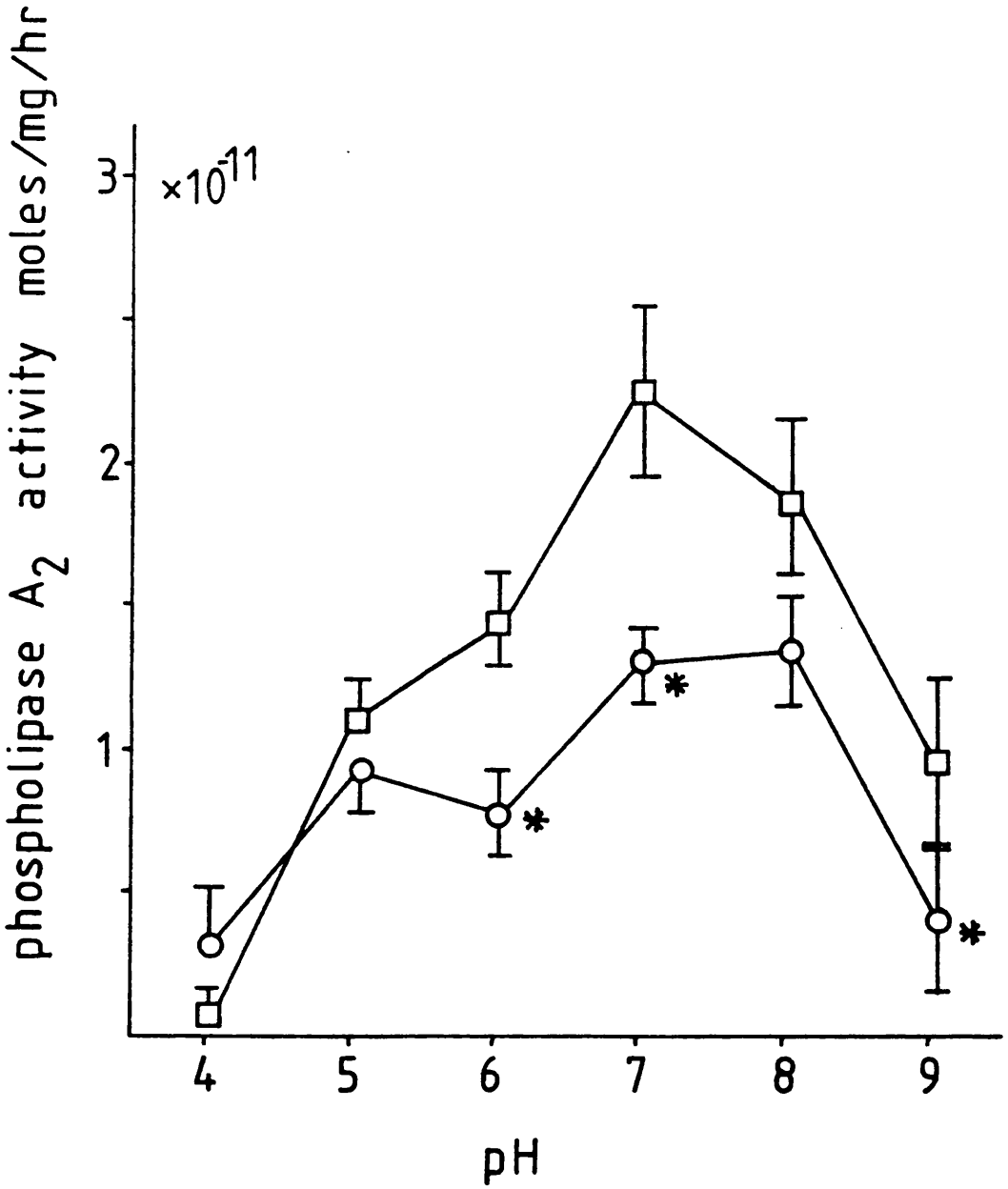


FIG 37. pH PROFILE OF PHOSPHOLIPASE A_2 ACTIVITY IN THE HOMOGENATE OF THE ISOLATED RAT HEART FOLLOWING 20 MINUTES CORONARY ARTERY LIGATION. MEAN $\pm$ SEM. n = 3.

□ NON-ISCHAEMIC AREA.
o ISCHAEMIC AREA.

* $p \leq 0.05$ vs NON-ISCHAEMIC AREA, PAIRED t-TEST.

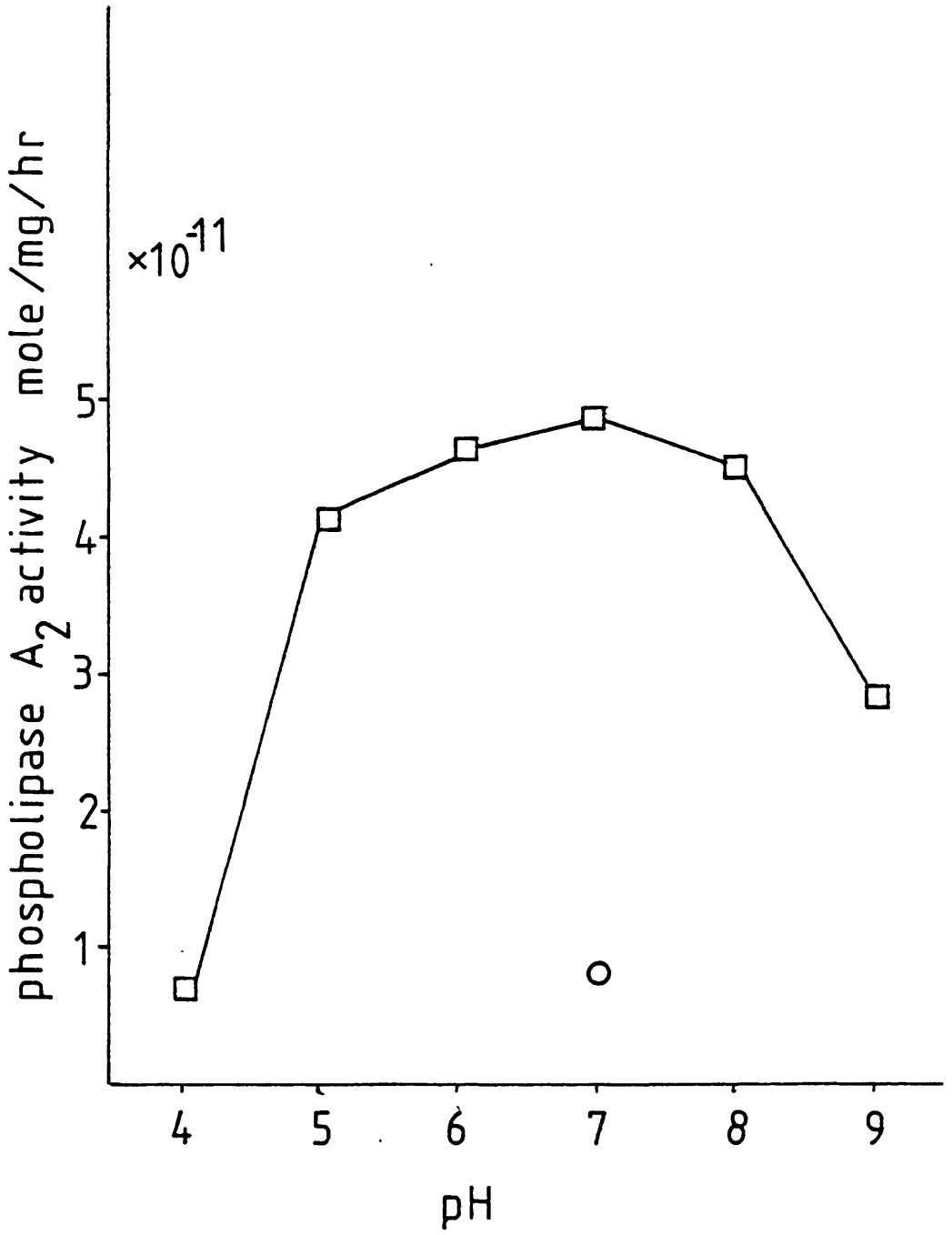


FIG 38. pH PROFILE OF MITOCHONDRIAL PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART.

□ 5mM Ca^{2+} , O 10mM EDTA.

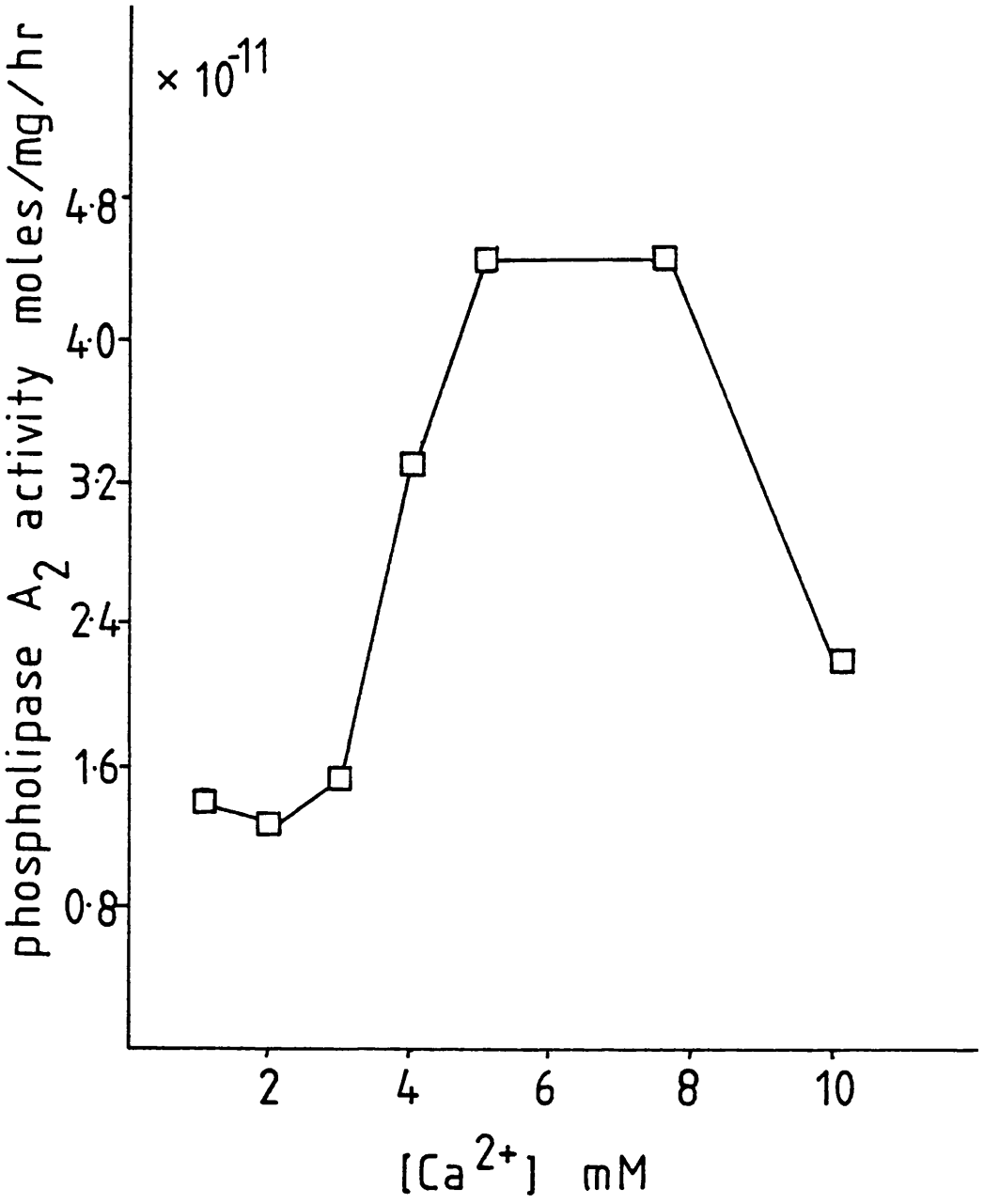


FIG 39. CALCIUM DEPENDENCE OF MITOCHONDRIAL PHOSPHOLIPASE A_2.

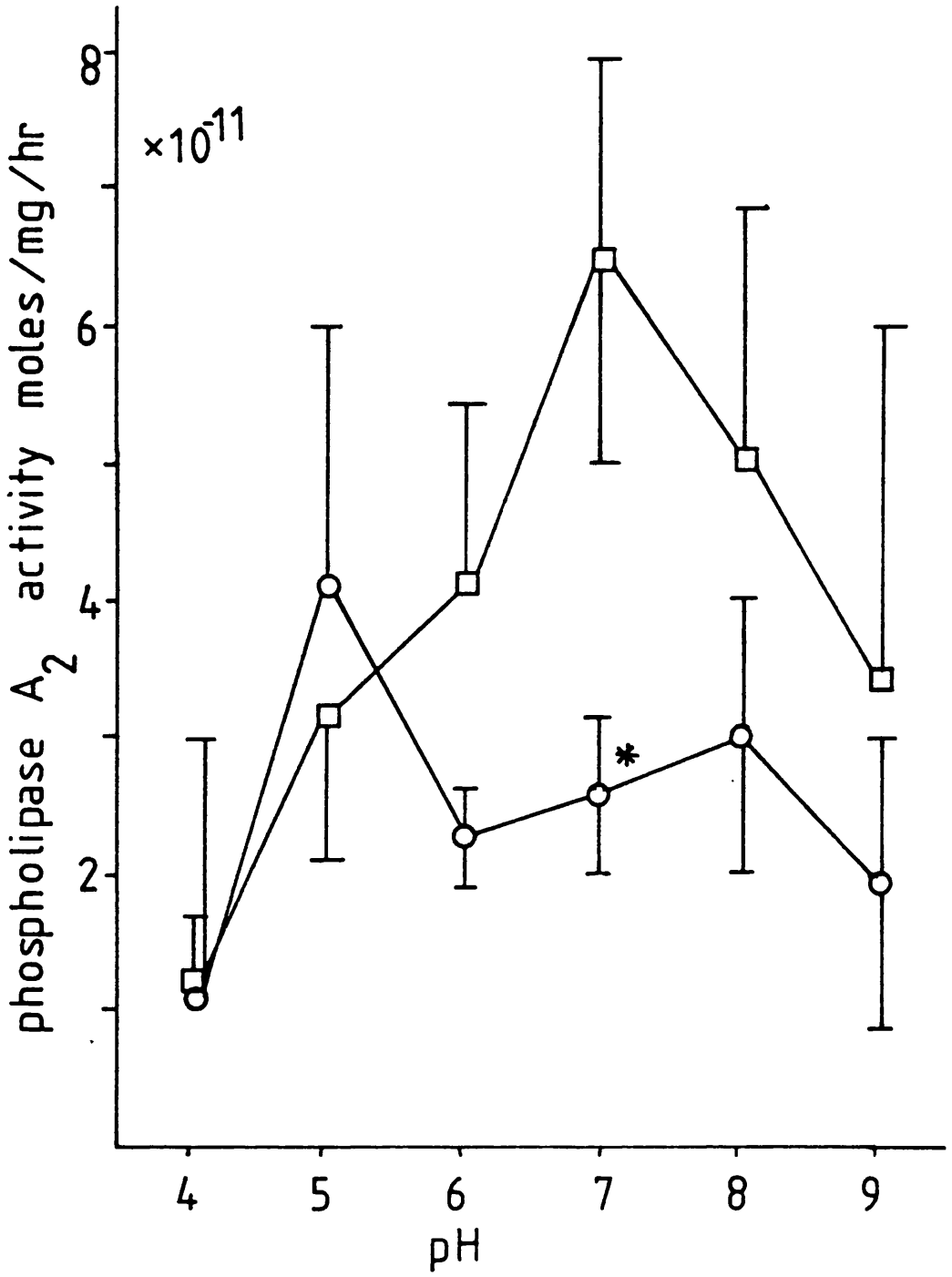


FIG 40. pH PROFILE OF MITOCHONDRIAL PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART FOLLOWING 20 MINUTES CORONARY ARTERY LIGATION. MEAN ± SEM. n = 4-6.

□ NON-ISCHAEMIC AREA.

○ ISCHAEMIC AREA.

* $p \leq 0.05$ vs NON-ISCHAEMIC AREA.

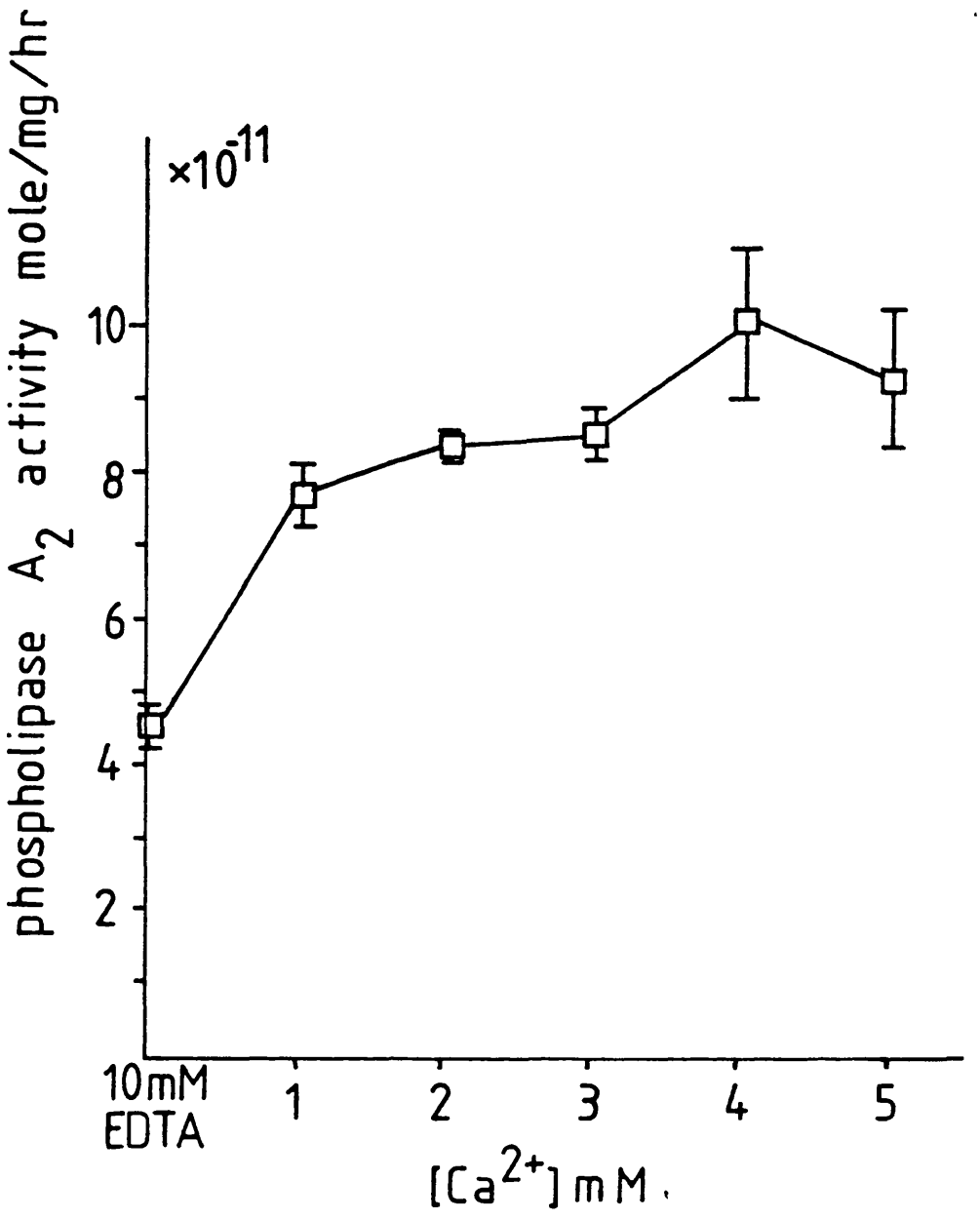


FIG 41. CALCIUM DEPENDENCE OF SARCOLEMMAL PHOSPHOLIPASE A_2 IN THE ISOLATED RAT HEART. MEAN $\pm$ SEM. n = 3.

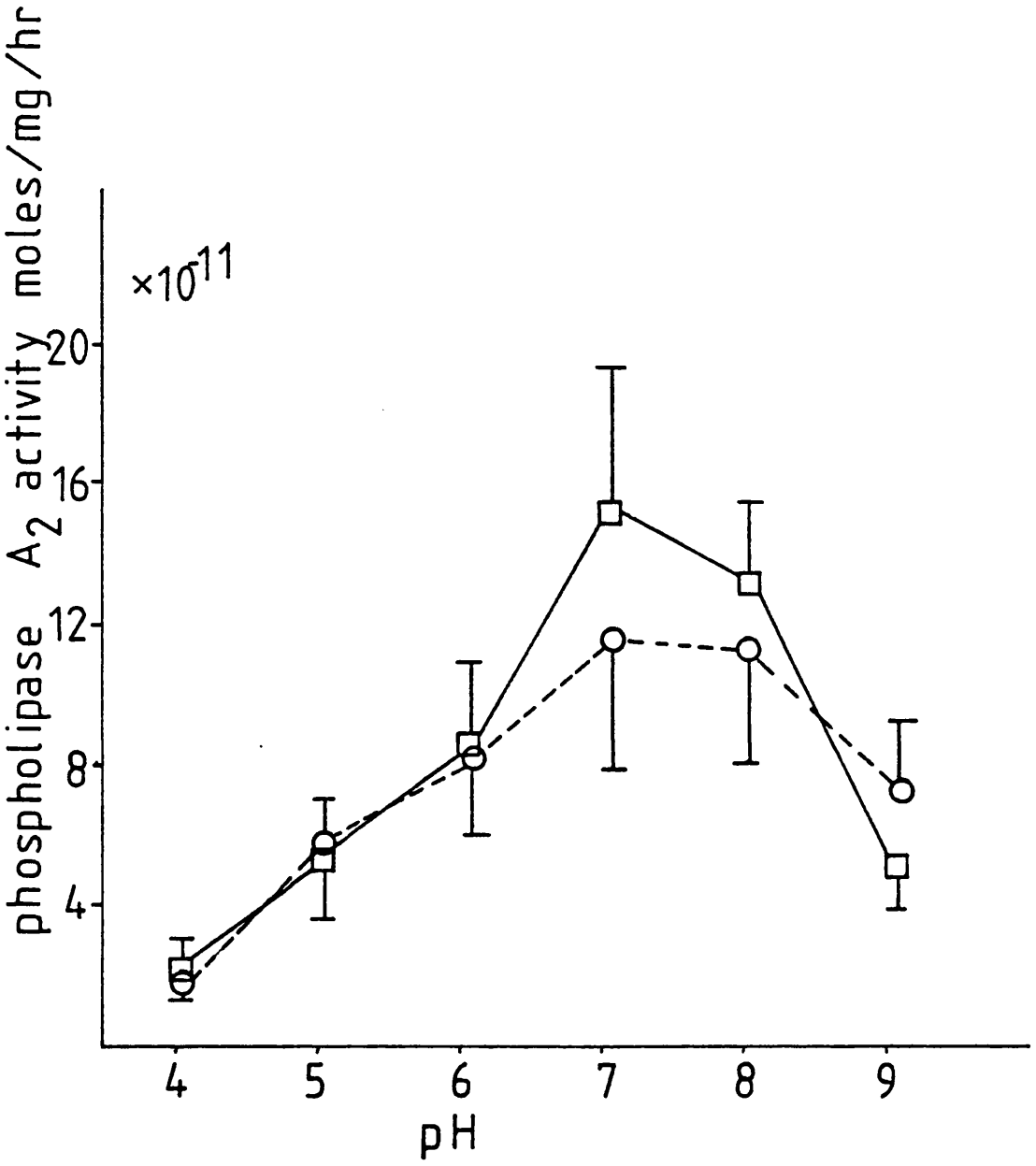


FIG 42. pH PROFILE OF SARCOLEMMAL PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART FOLLOWING 20 MINUTES CORONARY ARTERY LIGATION. MEAN ± SEM. n = 5.

□ NON-ISCHAEMIC AREA.
○ ISCHAEMIC AREA.

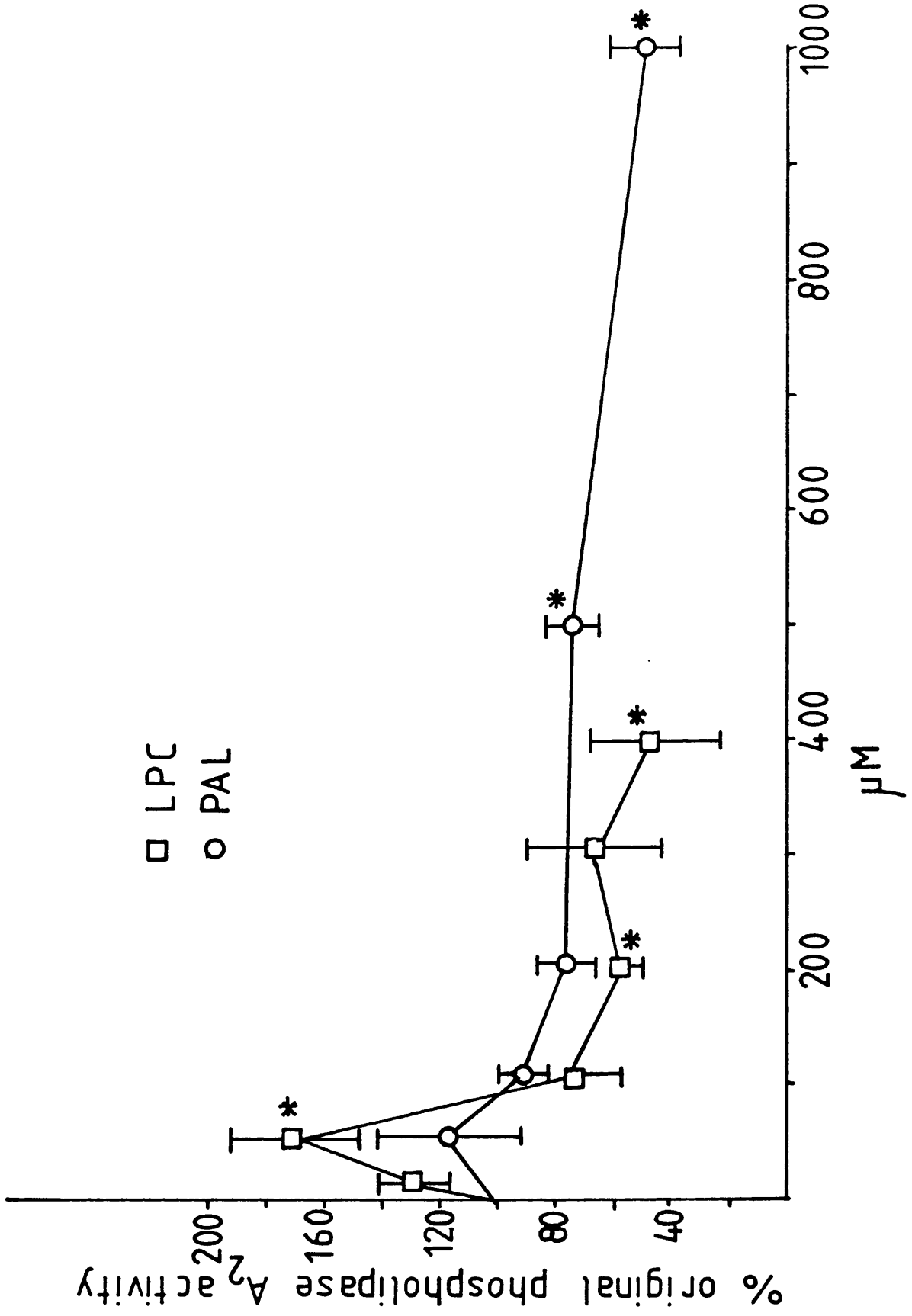


FIG 43. EFFECT OF LYSOPHOSPHATIDYLCHOLINE AND PALMITOYLCARNITINE ON PHOSPHOLIPASE A_2 ACTIVITY. MEAN $\pm$ SEM. n = 4.
* = $p \leq 0.05$ vs CONTROL.

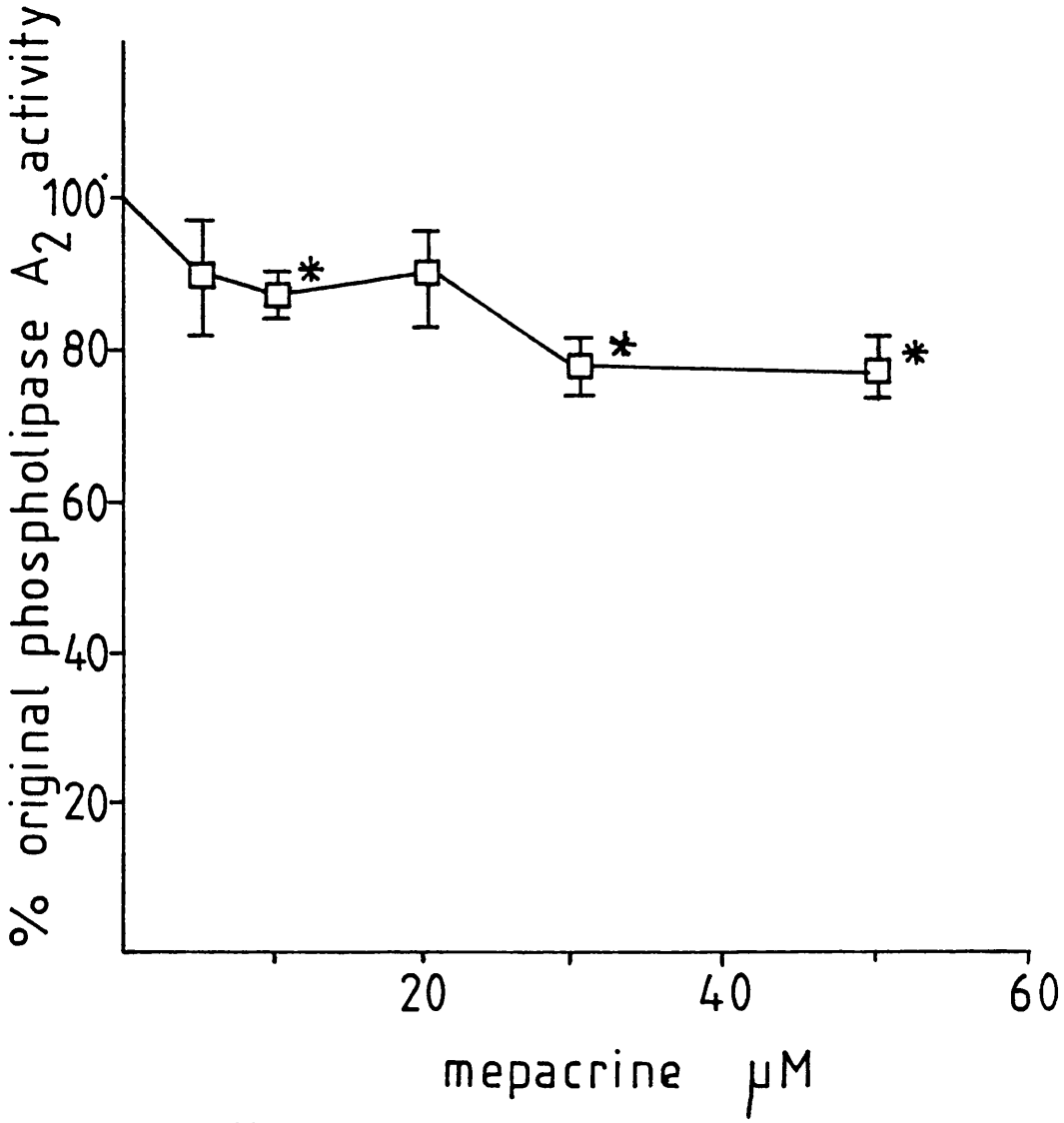


FIG 44. EFFECT OF MEPACRINE ON PHOSPHOLIPASE A_2 ACTIVITY. MEAN $\pm$ SEM. n = 5. * = $p \leq 0.05$ vs CONTROL.

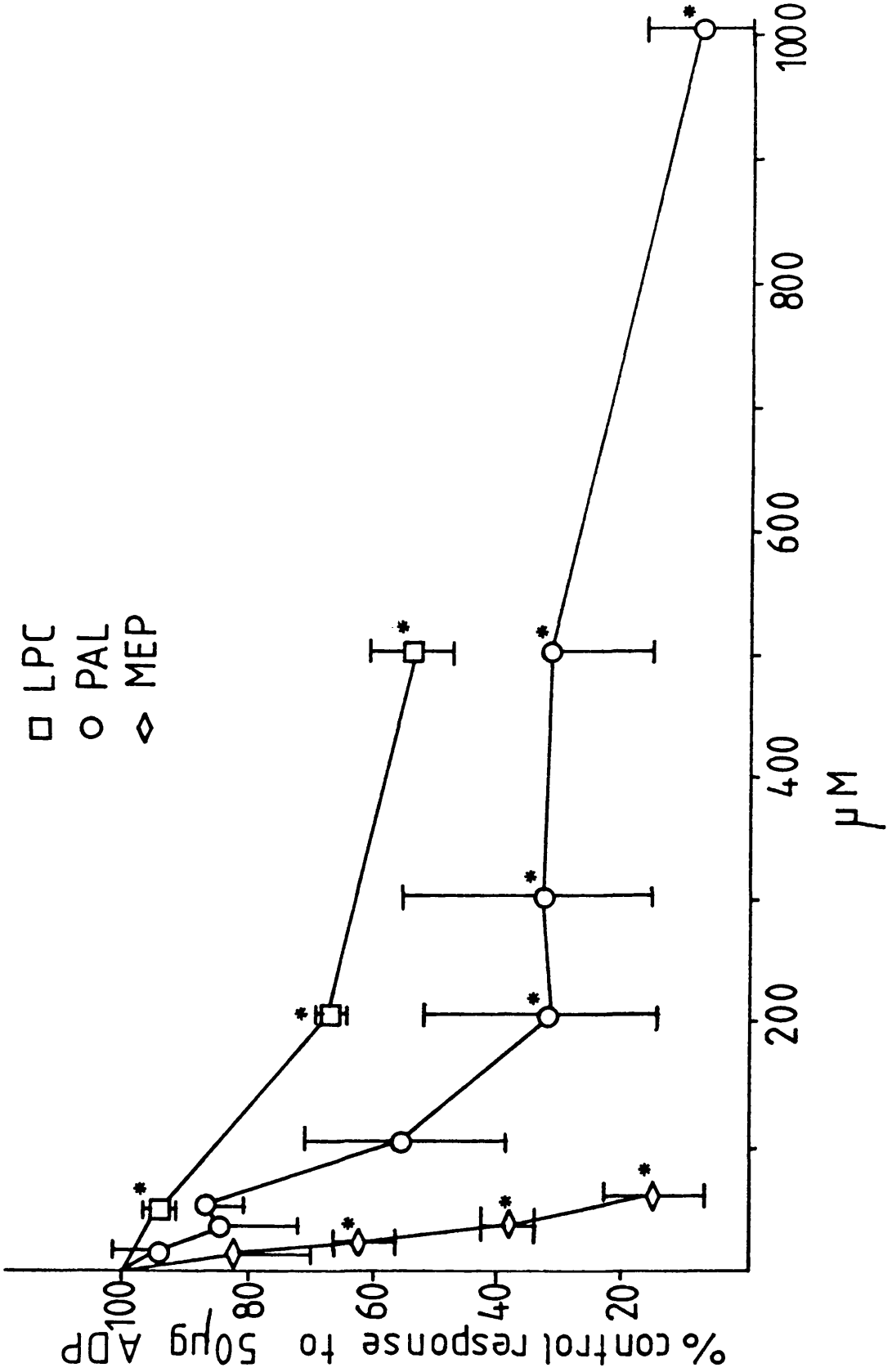


FIG 45. EFFECT OF LYSOPHOSPHATIDYLCHOLINE, PALMITOYLCARNITINE AND MEPACRINE ON ADP INDUCED PLATELET AGGREGATION. MEAN ± SEM. n = 3-5. * = $p \leq 0.05$ vs CONTROL.

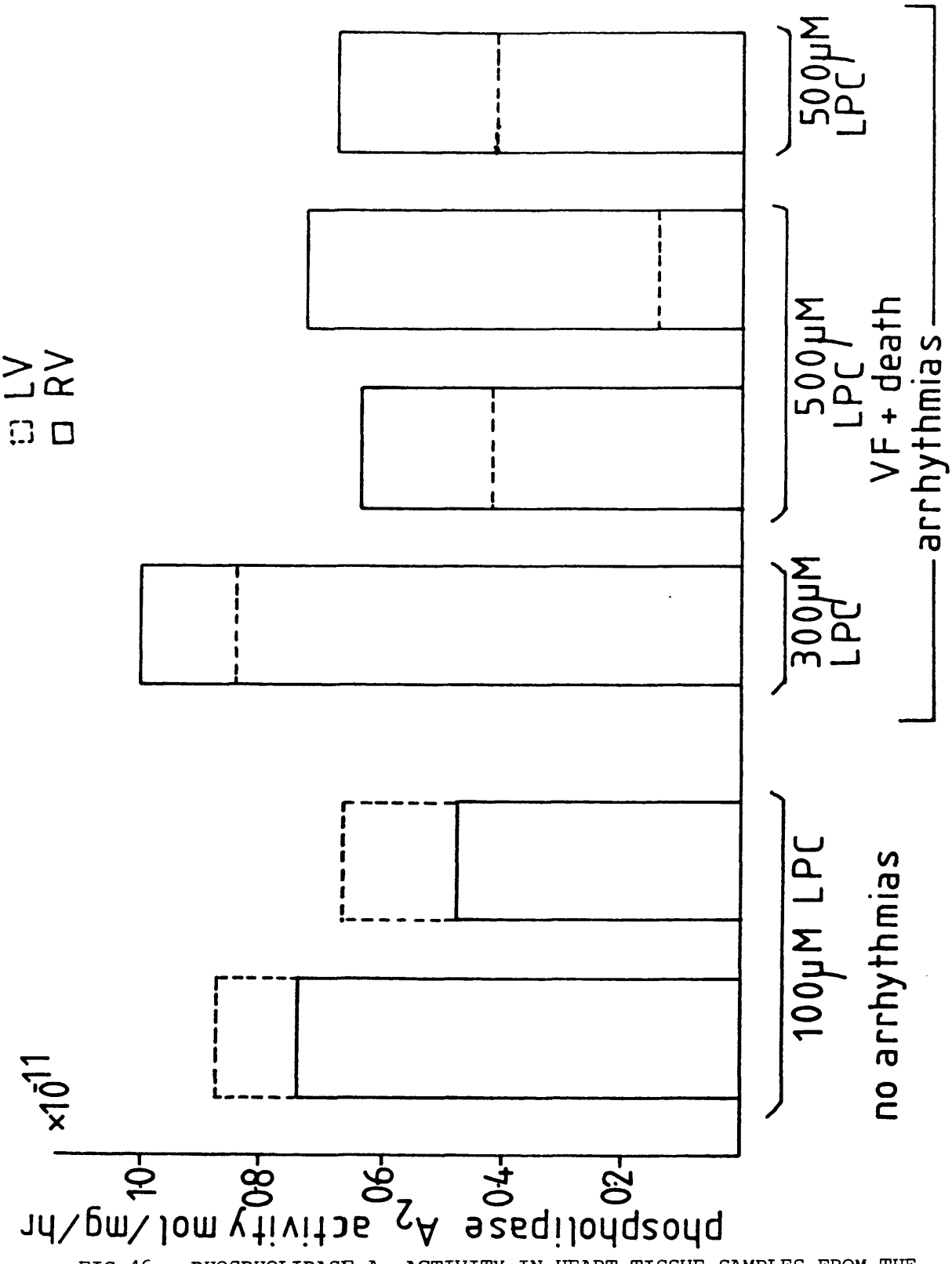


FIG 46. PHOSPHOLIPASE A_2 ACTIVITY IN HEART TISSUE SAMPLES FROM THE ANAESTHETIZED CAT FOLLOWING INTRACORONARY INFUSION OF LPC. LV = LEFT VENTRICULAR WALL. RV = RIGHT VENTRICULAR WALL.

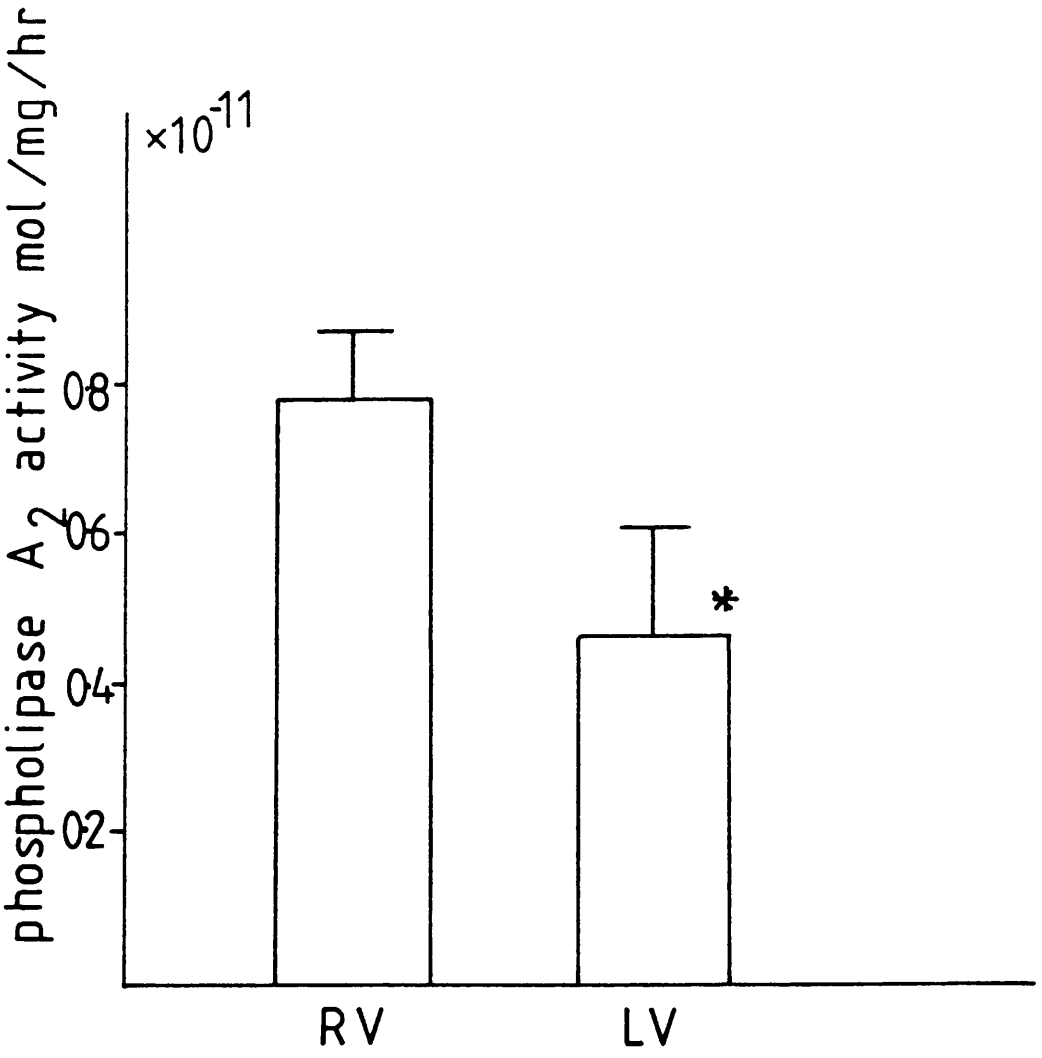


FIG 47. PHOSPHOLIPASE A_2 ACTIVITY IN THE LEFT (LV) AND RIGHT (RV) VENTRICULAR WALLS OF THE ANAESTHETIZED CAT IN HEARTS EXPERIENCING ARRHYTHMIAS FOLLOWING INTRA-CORONARY INFUSION OF LPC. MEAN $\pm$ SEM. n = 4. * = $p \leq 0.05$ vs RV. PAIRED t-TEST.

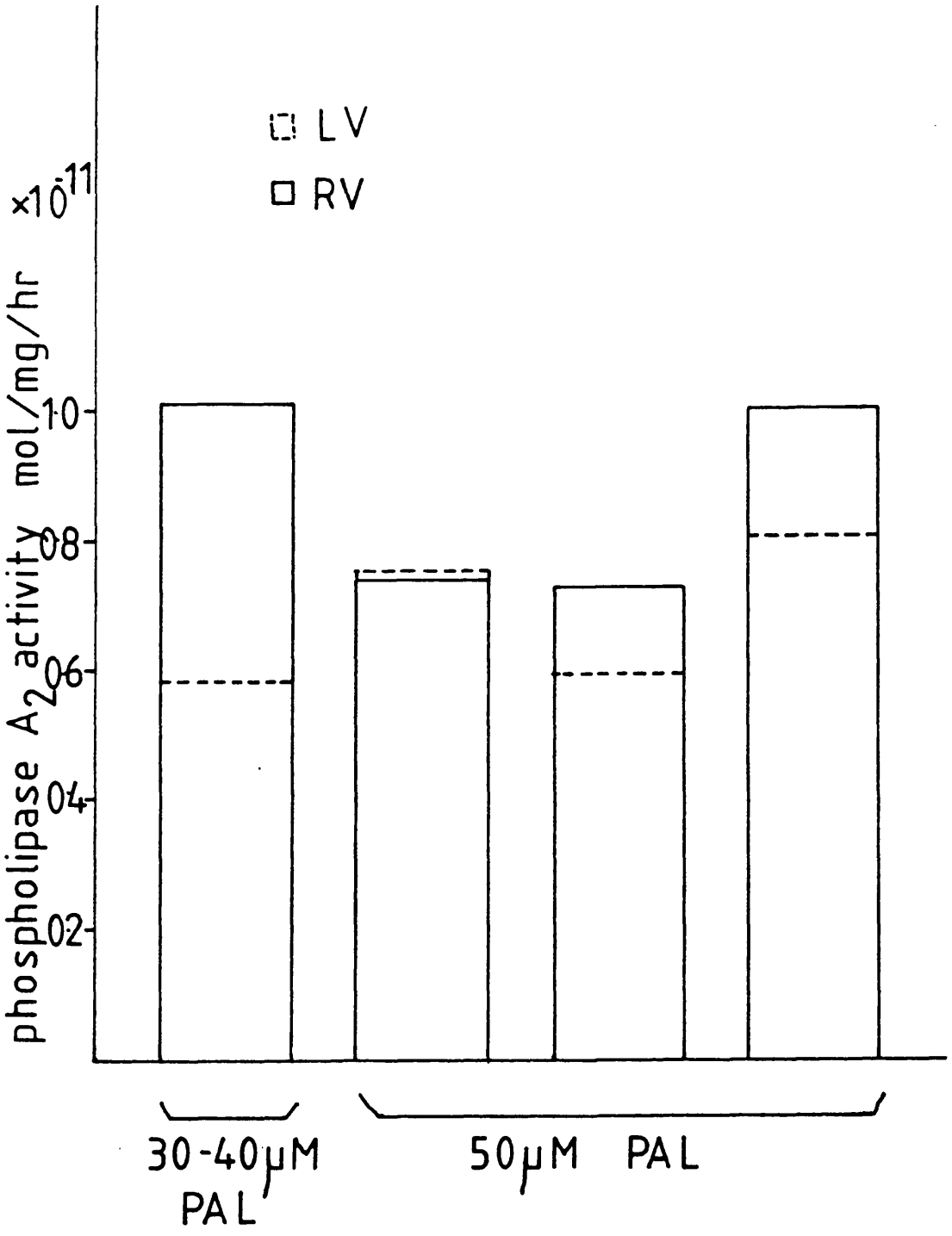


FIG 48. PHOSPHOLIPASE A_2 ACTIVITY IN HEART TISSUE SAMPLES FROM THE ANAESTHETIZED CAT FOLLOWING INTRACORONARY INFUSION OF PALMITOYLCARNITINE.

LV = LEFT VENTRICULAR WALL. RV = RIGHT VENTRICULAR WALL.

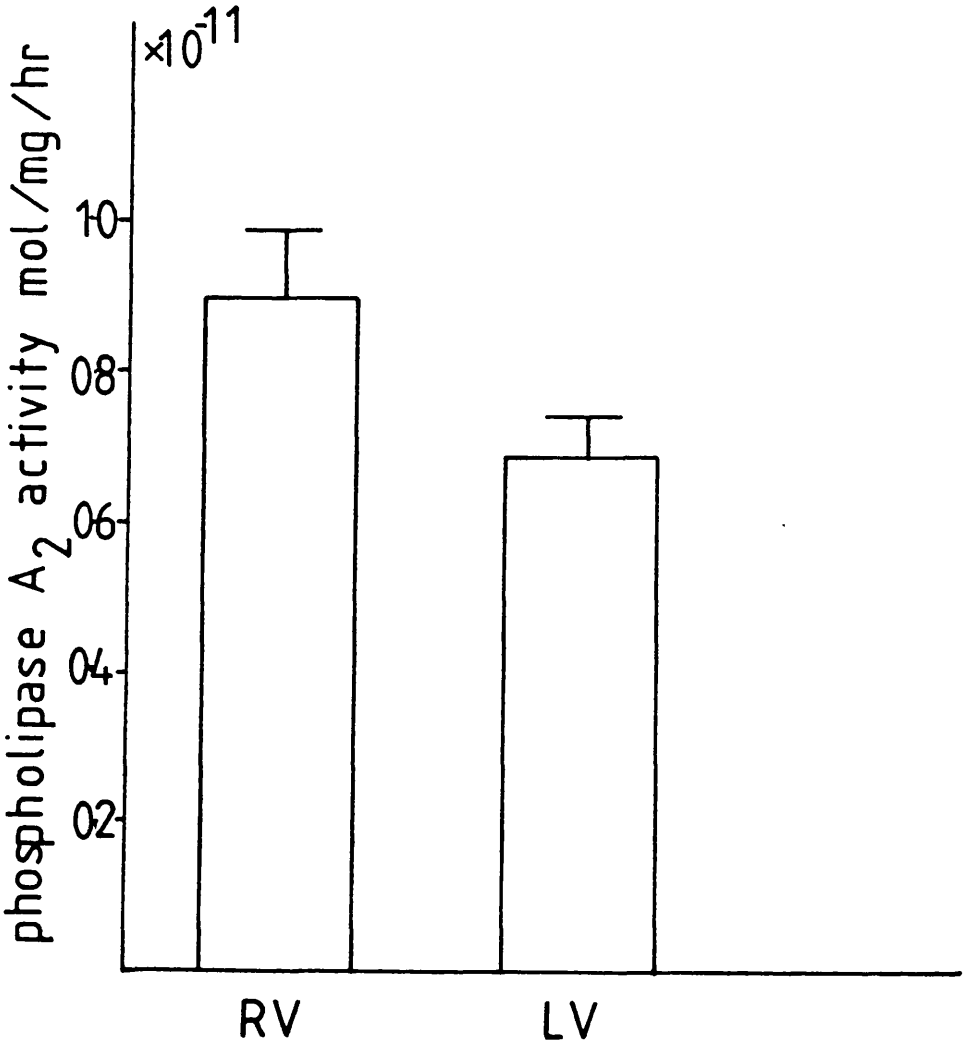


FIG 49. PHOSPHOLIPASE A_2 ACTIVITY IN THE LEFT (LV) AND RIGHT (RV) VENTRICULAR WALLS OF THE ANAESTHETIZED CAT IN HEARTS EXPERIENCING ARRHYTHMIAS FOLLOWING INTRACORONARY INFUSION OF PAL. MEAN $\pm$ SEM. n = 4.

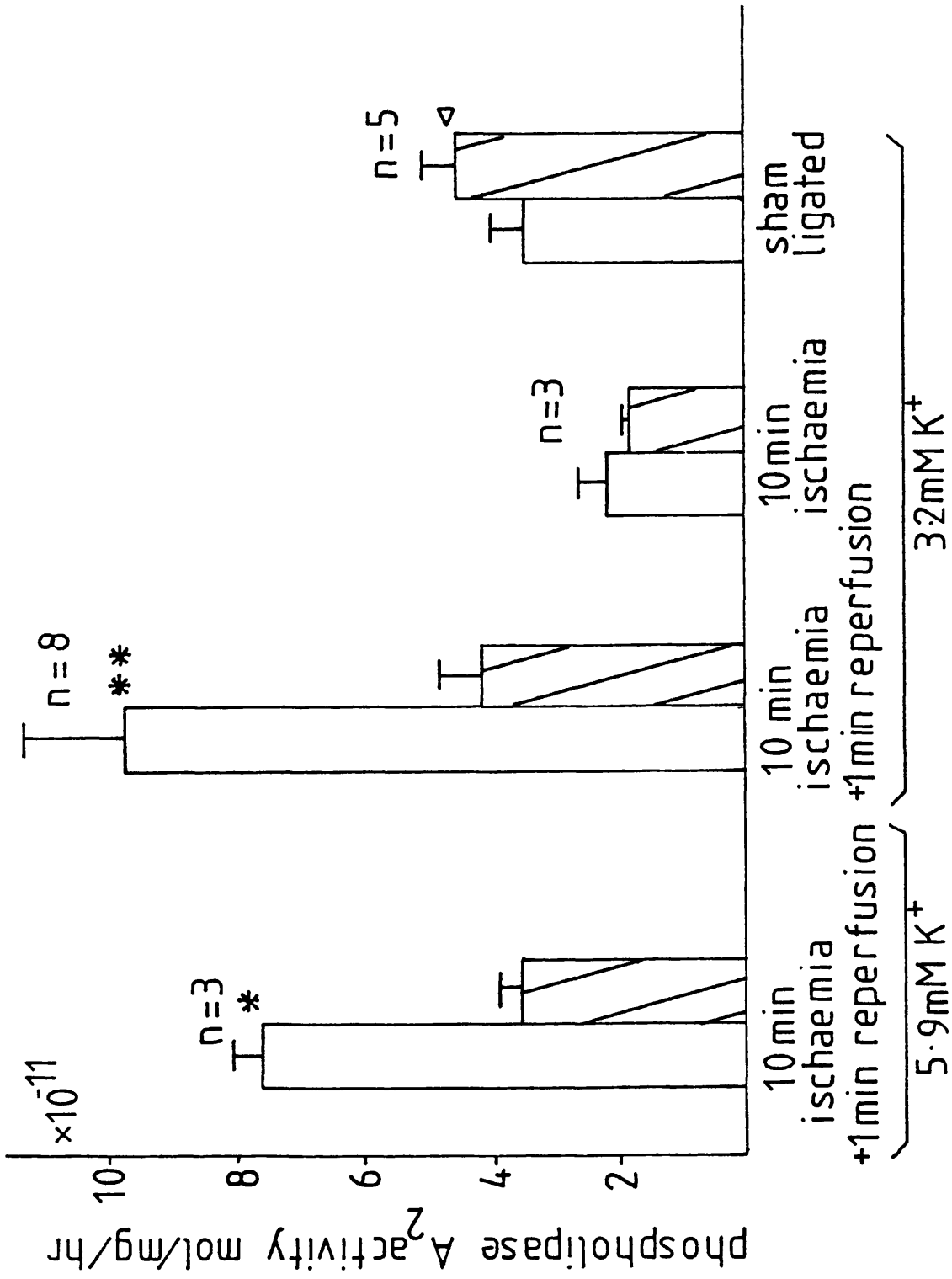


FIG 50. EFFECT OF 10 MINUTES CORONARY ARTERY OCCLUSION AND 1 MINUTE REPERFERUSION ON PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART USING DIFFERENT IONIC COMPOSITION BUFFERS. □ NON-ISCHAEMIC AREA. ▨ ISCHAEMIC AREA. MEAN ± SEM. * $p \leq 0.05$ vs ISCHAEMIC AREA, ** $p \leq 0.02$ vs ISCHAEMIC AREA, PAIRED t-TEST. ▽ $p \leq 0.01$ vs ISCHAEMIC AREA OF 10 MINS ISCHAEMIA ONLY.

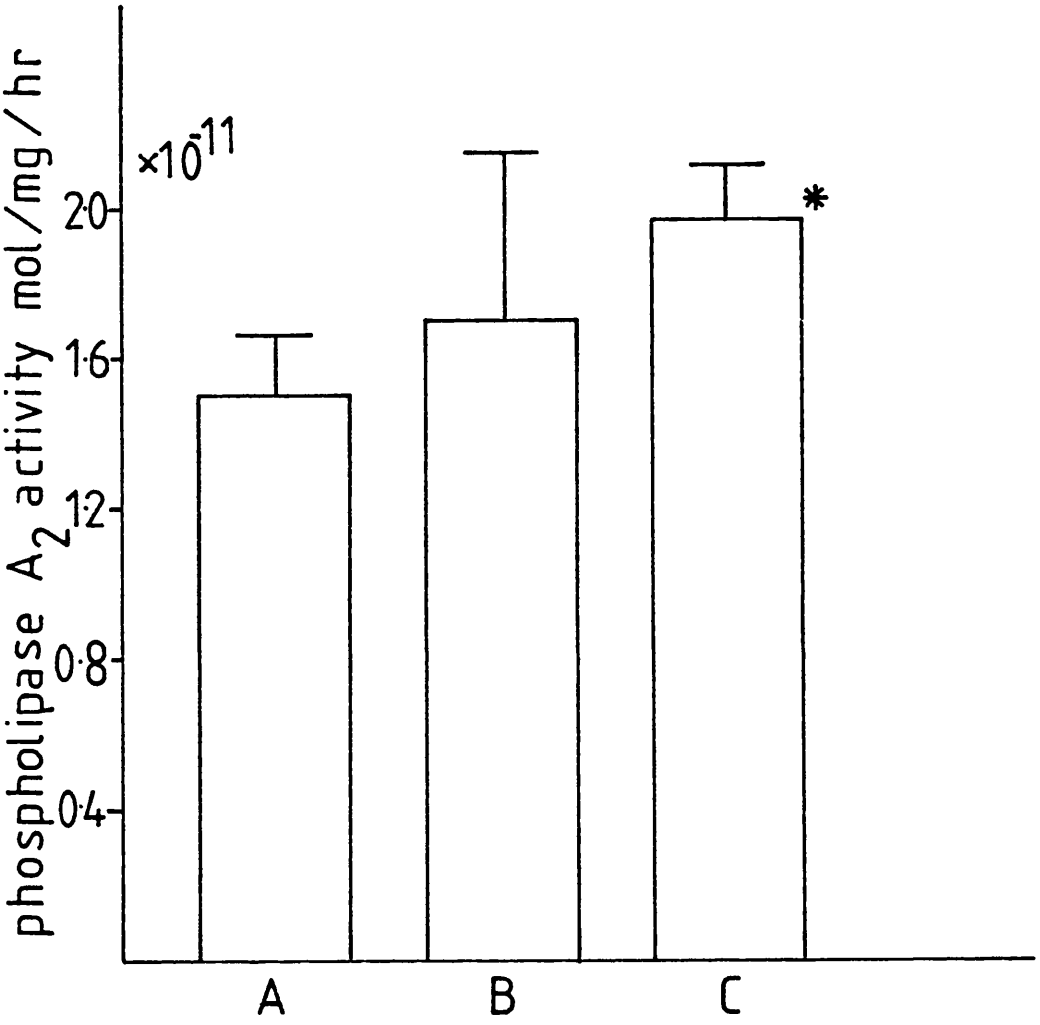


FIG 51. EFFECT OF PERFUSATE ELUTED FROM ISCHAEMIC AND REPERFUSED HEARTS ON PHOSPHOLIPASE A_2 ACTIVITY IN THE HOMOGENATE OF THE ISOLATED RAT HEART. MEAN ± SEM. n = 3. * $p \leq 0.05$ vs A, PAIRED t-TEST. A = CONTROL, B = PLUS ISCHAEMIC PERFUSATE, C = PLUS REPERFUSED PERFUSATE.

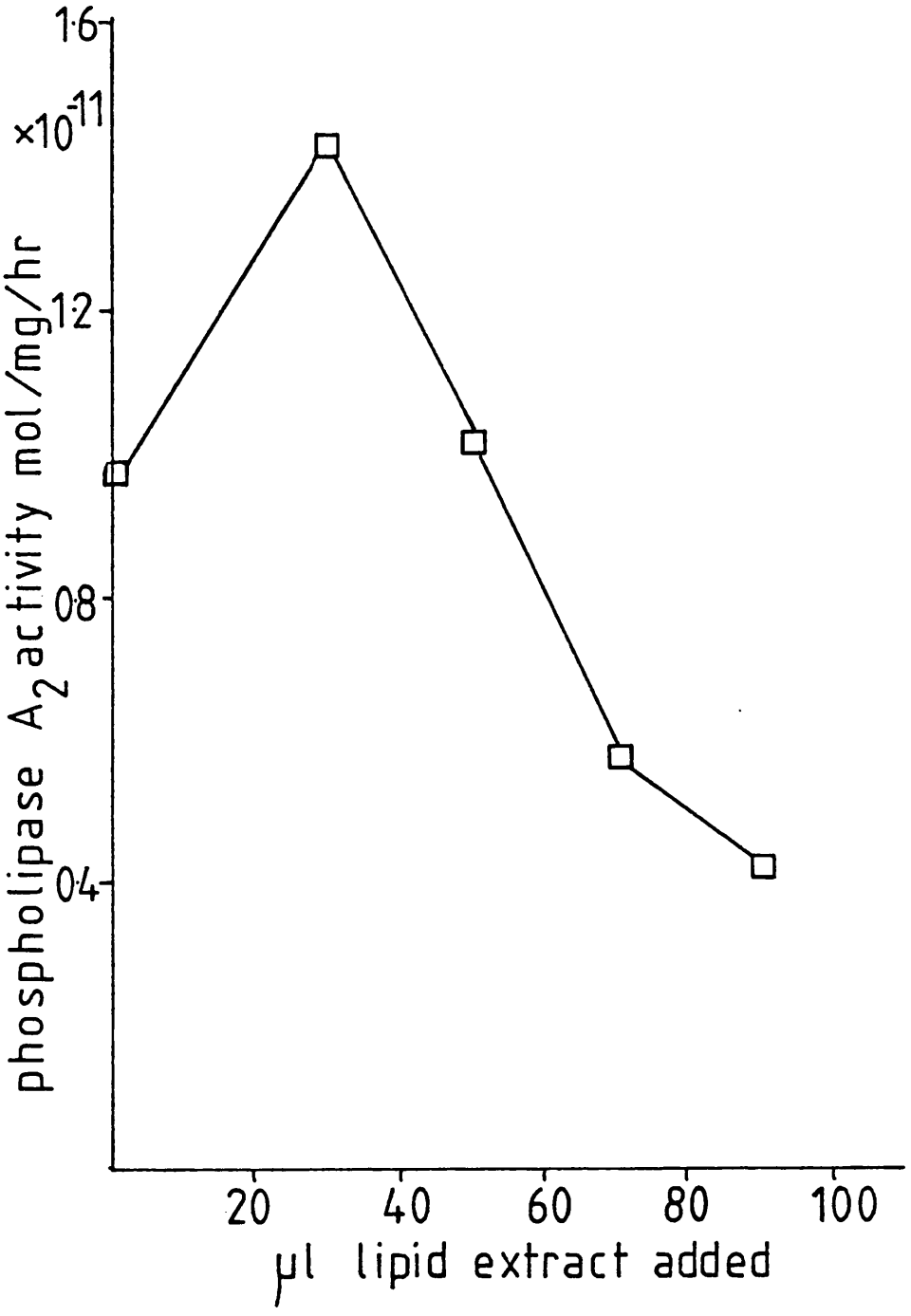


FIG 52. EFFECT OF LIPID EXTRACT OF PERFUSATE FROM A REPERFUSED HEART ON PHOSPHOLIPASE A_2 ACTIVITY IN A HOMOGENATE OF THE ISOLATED RAT HEART.

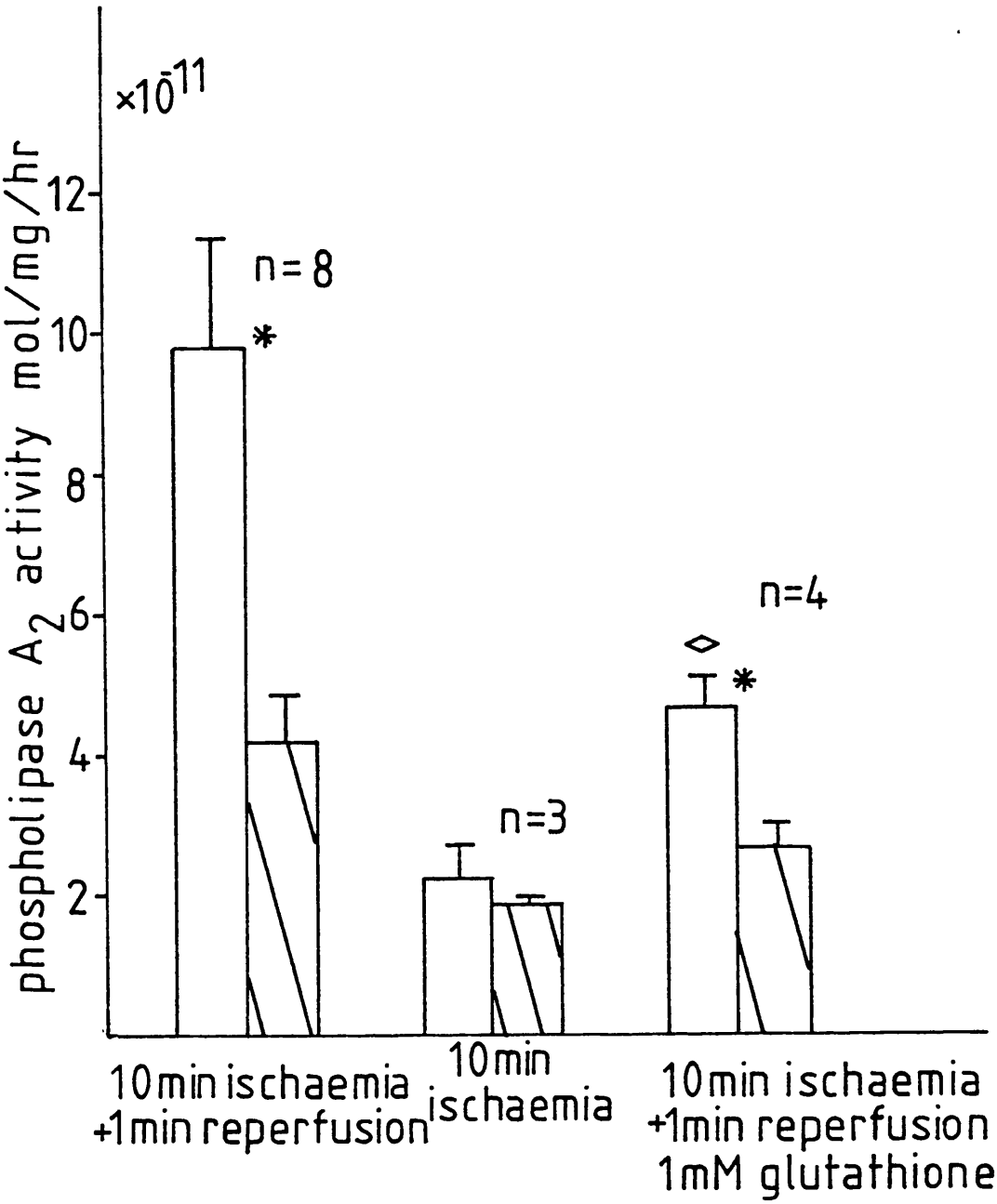


FIG 53. EFFECT OF 1mM REDUCED GLUTATHIONE ON PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART FOLLOWING 10 MINUTES CORONARY ARTERY LIGATION AND 1 MINUTE REPERFUSION. □ NON-ISCHAEMIC AREA. ▨ ISCHAEMIC AREA. MEAN ± SEM. * $p \leq 0.02$ vs ISCHAEMIC AREA, ◇ $p \leq 0.05$ vs NON-ISCHAEMIC AREA OF REPERFUSED HEART WITH NO GLUTATHIONE PRESENT.

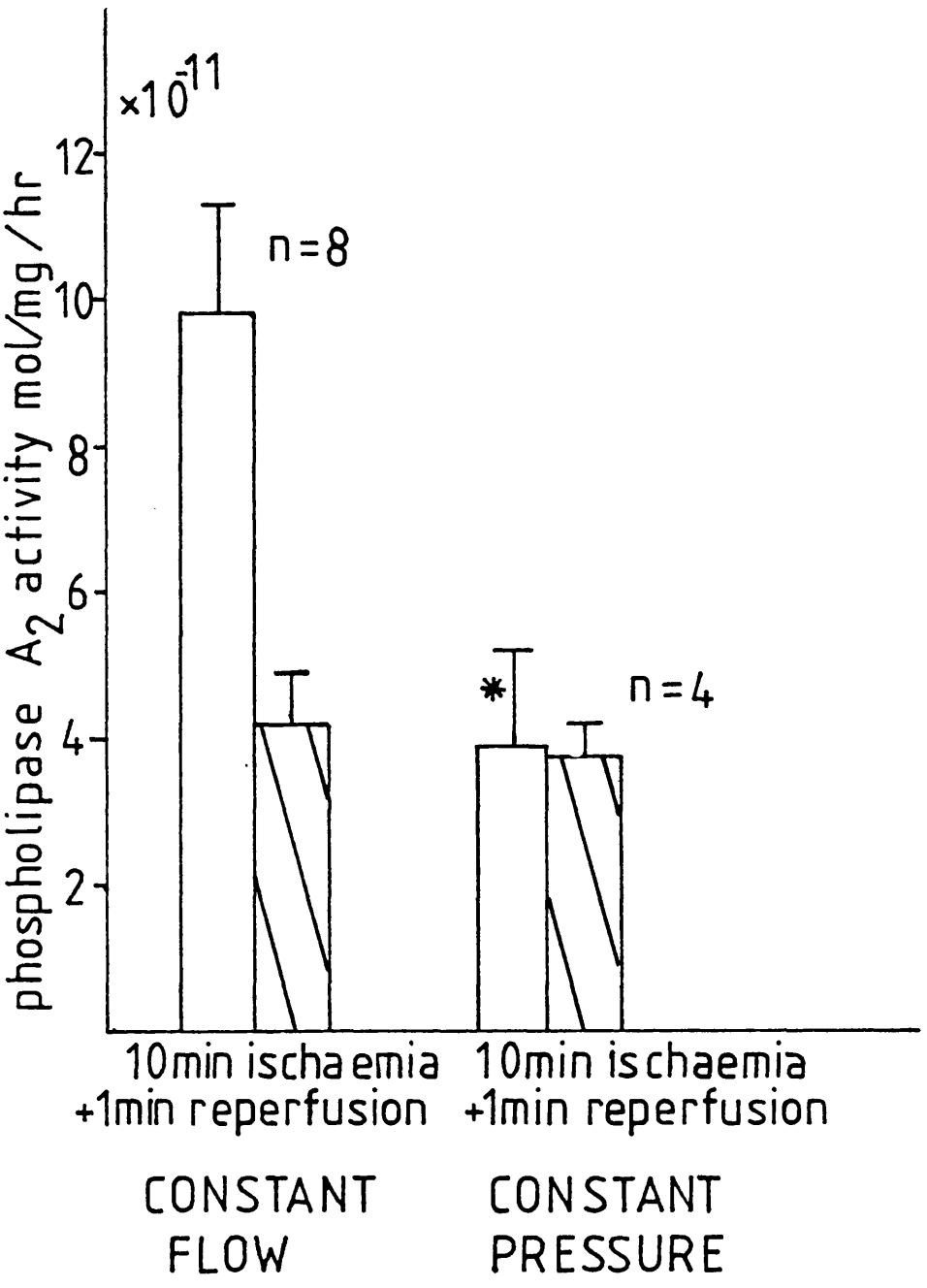


FIG 54. EFFECT OF A CONSTANT HEAD OF PRESSURE ON PHOSPHOLIPASE A_2 ACTIVITY IN THE ISOLATED RAT HEART FOLLOWING 10 MINUTES CORONARY ARTERY LIGATION AND 1 MINUTE REPERFUSION. □ NON-ISCHAEMIC AREA. ▨ ISCHAEMIC AREA. MEAN ± SEM. * $p \leq 0.05$ vs CONSTANT FLOW NON-ISCHAEMIC AREA.

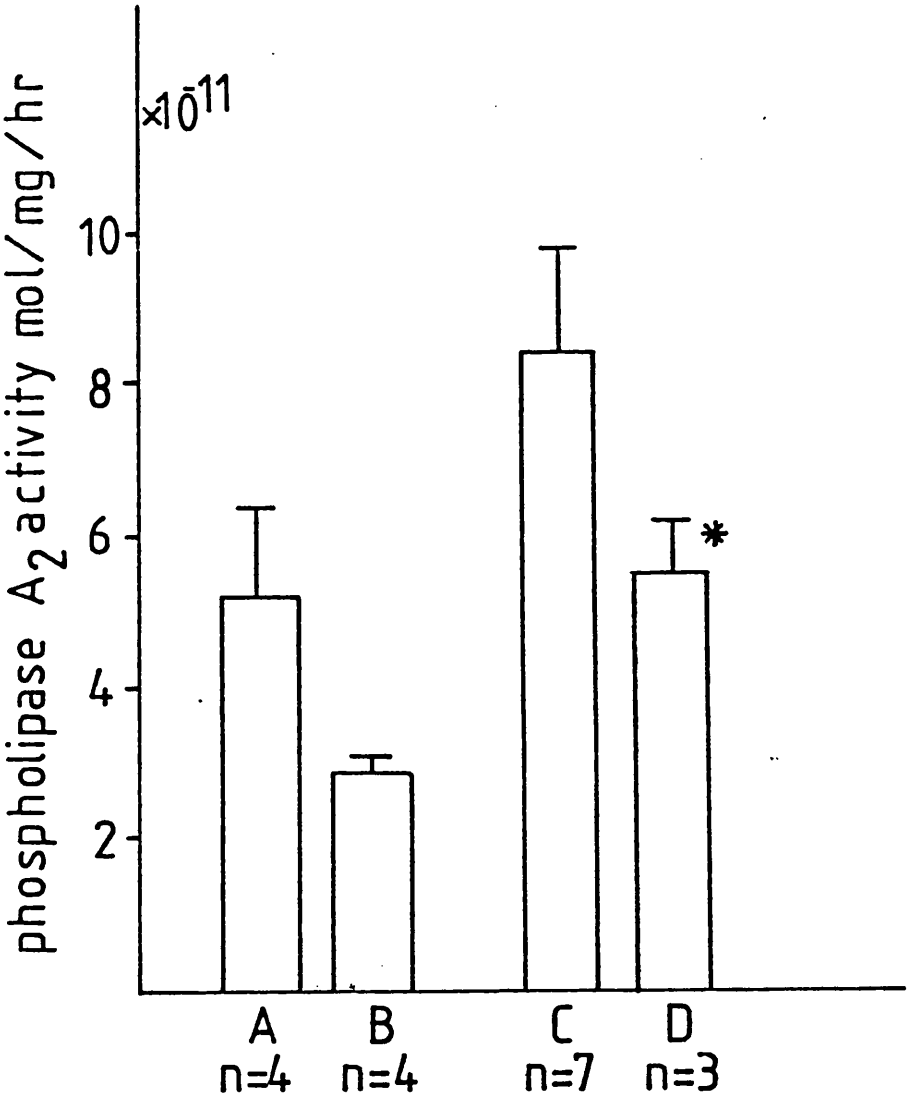


FIG 55. EFFECT OF FLOW CHANGES ON PHOSPHOLIPASE A_2 ACTIVITY IN A HOMOGENATE OF THE ISOLATED RAT HEART. MEAN ± SEM.
A = 10 MINS HIGH FLOW + 1 MIN NORMAL FLOW (SIMULATES THE NORMAL REGION OF THE REPERFUSED HEART).
B = 10 MINS NO FLOW + 1 MIN NORMAL FLOW (SIMULATES THE ISCHAEMIC REGION OF THE REPERFUSED HEART).
C = 10 MINS HIGH FLOW (SIMULATES THE NON-ISCHAEMIC REGION OF THE CORONARY ARTERY LIGATED HEART).
D = 10 MINS NO FLOW (SIMULATES THE ISCHAEMIC REGION OF THE CORONARY ARTERY LIGATED HEART).
* $p \leq 0.01$ vs B.

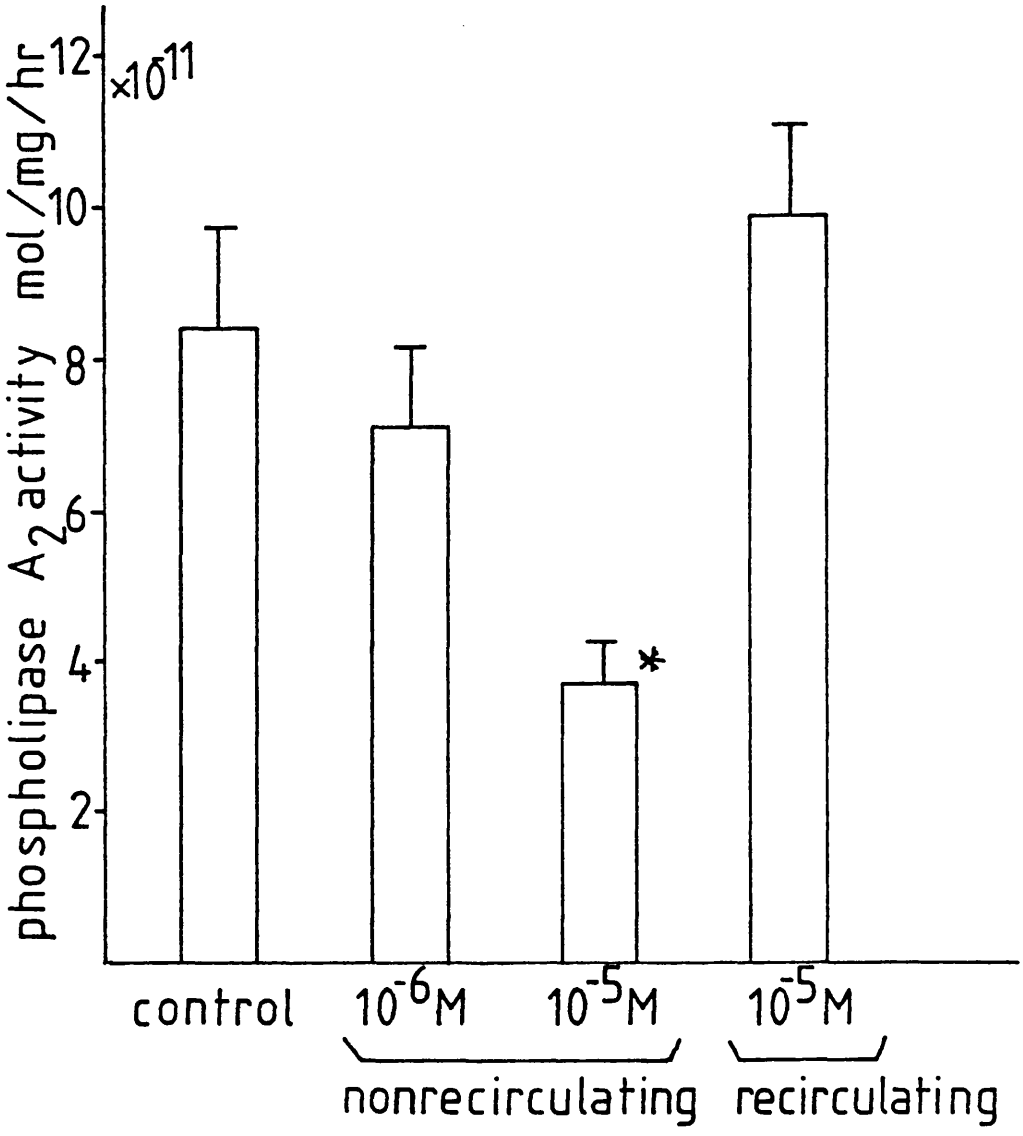


FIG 56. EFFECT OF ADENOSINE IN A RECIRCULATING AND A NON-RECIRCULATING SYSTEM ON THE PHOSPHOLIPASE A_2 ACTIVITY INCREASE PRODUCED BY HIGH FLOW PERFUSION. MEAN ± SEM. n = 3-4. * $p \leq 0.05$ vs CONTROL
VS ADENOSINE 10^{-6}M
$p \leq 0.01$ vs ADENOSINE 10^{-5}M (RECIRCULATING).

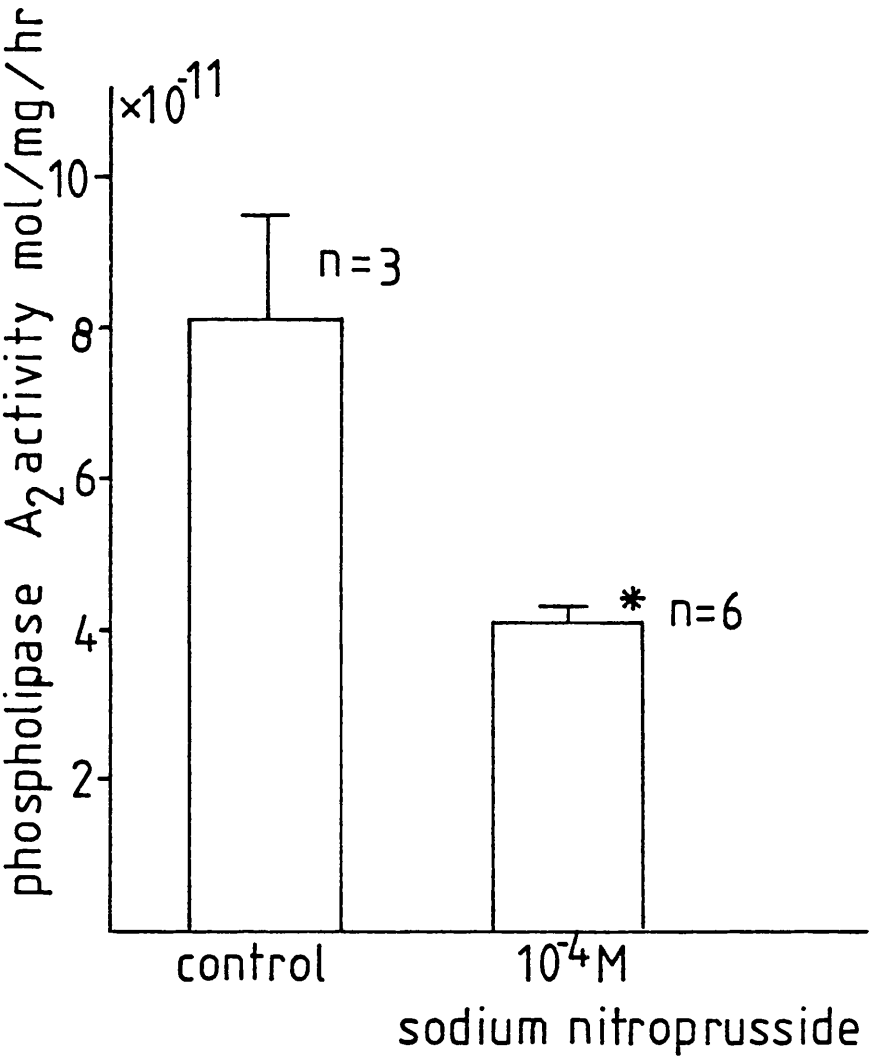


FIG 57. EFFECT OF SODIUM NITROPRUSSIDE ON THE PHOSPHOLIPASE A_2 ACTIVITY INCREASE PRODUCED BY HIGH FLOW PERFUSION. MEAN $\pm$ SEM.
* $p \leq 0.05$ vs CONTROL.

CONCLUSIONS

In the isolated rat heart coronary artery ligation was shown to produce an increase in lysophospholipid concentrations in the ischaemic area by five minutes post ligation. This concentration gradually increased over a 30 minute time course with the exception of the 25 minute time point, when concentrations were at control levels.

Lysophospholipid concentrations increased before the onset of arrhythmias, indicating that reported increased concentrations seen during ischaemia are unlikely to be actually produced by the arrhythmias. It is therefore possible that lysophospholipids have a role in the production of arrhythmias although the possibility exists that the two are independent of each other but have the same cause. A maximal lysophospholipid concentration appeared to be reached, possibly representing establishment of an equilibrium of its production and catabolism. Concentrations were also increased in the non-ischaemic area 10 minutes following coronary artery ligation. This could represent washout from the ischaemic area and recirculation to the non-ischaemic area.

Control hearts showed increases in lysophospholipid concentration when perfused with a buffer containing increased calcium or decreased potassium concentrations. Both of these ionic changes increase the severity of ischaemically induced arrhythmias. As lysophospholipids appear to be involved in the genesis of arrhythmias the

perfusate ionic changes may exacerbate ischaemically induced arrhythmias by increasing cellular lysophospholipid concentrations prior to any effects of ischaemia. There appeared to be a maximal change in lysophospholipid concentration that could be caused by either changes in perfusate ionic composition or coronary artery ligation. This may again represent an equilibrium between production and metabolism.

In the anaesthetized cat infusion of LPC into the coronary circulation was shown to be arrhythmogenic by a mechanism that did not appear to induce ischaemia. In contrast PAL was also arrhythmogenic but resulted in the production of ischaemia that could be reversed by the infusion of streptokinase. The time courses of both LPC and PAL induced arrhythmias were similar to that of coronary artery ligation. PAL may produce a vasoconstrictor effect potentiating the effect of any occlusive aggregates but the mechanism by which production of the latter occurs is unknown as PAL had no direct effects on platelet aggregation. It is possible that haemolysis or interaction with the endothelium occurs to produce pro-aggregatory substances.

The role of LPC in producing arrhythmias during ischaemia is supported by its production of arrhythmias in an *in vivo* normoxic model which is likely to be less sensitive to the electrophysiological derangements induced by LPC than ischaemic tissue.

Phospholipase A_2 activity was studied to determine its role in the production of LPC during ischaemia. At the time of maximal lysophospholipid concentration following coronary artery ligation PLA_2 activity was inhibited in the ischaemic area. As LPC and PAL were both shown to inhibit PLA_2 activity it is possible that the inhibition of PLA_2 seen during ischaemia is due to the accumulation of tnese two amphiphiles.

Mitochondrial PLA_2, in common with that measured in the homogenate, was reduced in activity following 20 minutes coronary artery ligation whilst sarcolemmal PLA_2 showed no difference in activity between the ischaemic and non-ischaemic areas. This may represent differences in the PLA_2 or washout of inhibitory substances from the sarcolemma during the isolation procedure.

PLA_2 activity was inhibited in the anaesthetized cat following infusion of LPC into the coronary artery to produce arrhythmias. This indicates that LPC concentrations capable of producing arrhythmias can lead to inhibited PLA_2. Although there was some inhibition of PLA_2 by PAL infusion this inhibition was not as great as with LPC, possibly due to the lower concentrations of PAL needed to produce arrhythmias. No difference was seen in PLA_2 activity in the ischaemic and non-ischaemic areas following coronary artery ligation. This may be due to washout of inhibitory substances from the ischaemic

region as the collateral flow in the cat is substantially greater than in the rat heart and blood perfusion will enhance the washout of LPC in comparison to Krebs-Henseleit perfusion.

Reperfusion of the isolated rat heart produced an increase in PLA_2 activity in the non-ischaemic area and to a lesser extent in the ischaemic area. This may be partially due to flow changes as some of the PLA_2 activity changes could be produced by simulation of flow rate changes in whole hearts. Free radical production and lipid peroxidation may also activate PLA_2 by interacting with the lipid membranes and may be potentiated by lipid ampniphiles produced during ischaemia.

REFERENCES

Ahumada G.G., Bergmann S.R., Carlson E, Corr P.B. and Sobel B.E. (1979). Augmentation of cyclic AMP content induced by lysophosphatidylcholine in rabbit hearts. Cardiovasc. Res. 13, 377-382.

Allen D.G., Eisner D.A. and Orchard C.H. (1984). Factors influencing free intracellular calcium concentration in quiescent ferret ventricular muscle. J. Physiol. 350, 615-630.

Allison T.B., Ramey C.A. and Holsinger J.W. (1977). Transmural gradients of left ventricular metabolites after circumflex artery ligation in dogs. J.Mol.Cell.Cardiol. 9, 837-852.

Anversa P., Olivetti G., Melissari M. and Loud A.V. (1980). Stereological measurement of cellular and subcellular hypertrophy and hyperplasia in the papilliary muscle of the adult rat. J.Mol.Cell.Cardiol. 12, 781-795.

Armstrong A., Duncan B., Oliver M.F., Julian D.G., Donald K.W., Fulton M., Lutz W. and Morrison S.L. (1972). Natural history of acute coronary heart attacks. A community study. Br.Heart.J. 34, 67-80.

Arnsdorf M.F. and Sawicki G.J. (1981). The effects of lysophosphatidylcholine, a toxic metabolite of ischaemia, on the components of cardiac excitability in sheep Purkinje fibres. Circ. Res. 49, 16-30.

Arthur G. and Choy P.C. (1986). Acylation of 1-alkenyl-glycerophosphocholine and 1-acyl-glycerophosphocholine in guinea pig heart. Biochem.J. 236, 481-487.

Arthur G., Page L., Mock T. and Choy P.C. (1986). The catabolism of plasmenylcholine in the guinea pig heart. Biochem.J. 236, 475-480.

Azuma T. and Fukushima T. (1976). Disturbance of blood flow as a factor in thrombus formation. Thromb.Res. 8, supp. II, 375-380.

Bagdonas A.A., Stukey J.H., Piera J., Amer N.S. and Hoffman B.F. (1961). Effects of ischaemia and hypoxia on the specialised conducting system of the canine heart. Am.Heart.J. 61, 206-218.

Ballou L.R. and Cheung W.Y. (1985). Inhibition of human platelet phospholipase A_2 activity by unsaturated fatty acids. Proc.Natl.Acad.Sci. 82,371-375.

Bartlett G.R. (1959). Phosphorus assay in column chromatography. J.Biol.Chem. 234, 466-468.

Bell R.L., Kennerly D.A., Stanford N. and Majerus P.W. (1979). Diglyceride lipase: A pathway for arachidonate release from human platelets. Proc.Natl.Acad.Sci. 76, 3238-3241.

Bencini D.A., Wild J.R. and O'Donovan G.A. (1983). Linear one step assay for the determination of orthophosphate. Anal.Biochem. 132,254-258.

Bergmann W.L., Dressler V., Haest C.W.M. and Deuticke B. (1984). Cross linking of SH groups in the erythrocyte membrane enhances transbilayer reorientation of phospholipids. Evidence for a limited access of phospholipids to the reorientation sites. Biochim.Biophys.Acta. 769, 390-398.

Bergmann S.R., Ferguson T.B. and Sobel B.E. (1981). Effects of amphiphiles on erythrocytes, coronary arteries and perfused hearts. Am.J.Physiol. 240, H229-H237.

Berlin J.R., Cannell M.B., Goldman W.F., Lederer W.J., Marban E. and Wier W.G. (1985). Subcellular calcium inhomogeneity indicated by fura-2 develops with calcium overload in single rat heart cells. Prog.Physiolog.Soc. Sept. 1985.

Berne R.M. (1980). The role of adenosine in the regulation of coronary blood flow. Circ.Res. 47, 807-813.

Bernheim F., Bernheim M.L.C. and Wilbur K.M. (1948). The reaction between thiobarbituric acid and the oxidation products of certain lipids. J.Biol.Chem. 174, 257-264.

Berridge M.J. (1984). Inositol phosphate and diacylglycerol as second messengers. Biochem.J. 220, 345-360.

Bersohn M.M. and Philipson K.D. (1983). Lysophosphoglycerides and sodium-calcium exchange in cardiac sarcolemma. Circulation. 68,supp III, 667.

Bersohn M.M., Philipson K.D. and Fukushima J.Y. (1982). Sodium-calcium exchange and sarcolemmal enzymes in ischaemic rabbit hearts. Am.J.Physiol. 242, C288-C295.

Bigaud M. and Spedding M. (1986). Inhibition of the effects of endothelial-derived relaxant factor (EDRF) in aorta by palmitoylcarnitine. Br.J.Pharmac. 89, 540P.

Bills T.K., Smith J.B. and Silver M.J. (1976). Metabolism of C^{14} arachidonic acid by human platelets. Biochim.Biophys.Acta. 424, 303-314.

Bird R.P. and Draper H.H. (1984). Comparative studies on different methods of malondialdehyde determination. Methods in Enzymology 105, 299-305.

Bjørnstad P. (1966). Phospholipase activity in rat liver mitochondria studied by the use of endogenous substrates. J.Lipid. Res. 7, 612-620.

Blackwell G.J., Duncombe W.G., Flower R.J., Parsons M.F. and Vane J.R. (1977). The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs. Br.J.Pharmac. 59, 353-366.

Bloor C.M. and White F.C. (1982). Approaches to the containment of myocardial-infarct size following coronary-vessel occlusion. In: Kalsner S. ed. The

coronary artery, pp 685-706. London: Croom Helm Ltd.

Braasch W., Gudbjarnason S., Puri P.S., Ravens K.G. and Bing R.J. (1968). Early changes in energy metabolism in the myocardium following acute coronary artery occlusion in anaesthetized dogs. Circ.Res. 23, 429-438.

Branwood A.W. (1978). The development of coronary thrombosis following myocardial infarction. Lipids. 13, 378-379.

Bricknell O.L. and Opie L.H. (1978). Effects of substrates on tissue metabolic changes in the isolated rat heart during underperfusion and on release of lactate dehydrogenase and arrhythmias during reperfusion. Circ.Res. 43, 102-115.

Bridge J.H.B. and Bassingthwaite J.B. (1982). Uphill sodium transport driven by an inward calcium gradient in heart muscle. Science. 219, 178-180.

Burke S.E., Antonaccio M.J. and Lefer A.M. (1982). Lack of thromboxane A_2 involvement in the arrhythmias occuring during acute myocardial ischaemia in dogs. Basic.Res.Cardiol. 77, 411-422.

Carlsson L., Abrahamsson T. and Almgren O. (1985). Local release of myocardial norephinephrine during acute ischaemia: An experimental study in the isolated perfused rat heart. J.Cardiovasc.Pharmac. 7, 791-798.

Ceremuzynski L. (1981). Hormonal and metabolic reactions evoked by acute myocardial infarction. Circ.Res. 48, 767-776.

Chapman D., Urbina J. and Keough K.M. (1974). Biomembrane phase transitions. Studies of lipid-water systems using differential scanning calometry. J.Biol.Chem. 249, 2512-2521.

Chen S.S-H. and Kou A.Y. (1982). Improved procedure for the separation of phospholipids by high performance liquid chromatography. J.Chromatog. 227, 25-31.

Chen S.S-H. Kou A.Y. and Chen H.H-Y. (1981). Measurement of ethanolamine and serine-containing phospholipids by high performance liquid chromatography with fluorescence detection of their Dns derivatives. J.Chromatog. 208, 339-346.

Cheung J.Y., Bonventre J.V., Malis C.D. and Leaf A. (1986). Calcium and ischaemic injury. New.Eng.J.Med. 314, 1670-1676.

Cheung J.Y., Leaf A. and Bonventre J.V. (1984). Mechanism of protection by verapamil and nifedipine from anoxic injury in isolated cardiac myocytes. Am.J.Physiol. 246, C323-C329.

Chien K.R., Abrams J., Serroni A., Martin J.T. and Farber J.L. (1978). Accelerated phospholipid degradation and associated membrane dysfunction in irreversible liver cell injury. J.Biol.Chem. 253, 4809-4817.

Chien K.R., Han A., Sen A., Buja M. and Willerson J.T. (1984). Accumulation of unesterified arachidonic acid in ischaemic canine myocardium - Relationship to a phosphatidylcholine deacylation-reacylation cycle and the depletion of membrane phospholipids. Circ.Res. 54, 313-322.

Clarkson C.W. and Ten Eick R.E. (1983). On the mechanism of lysophosphatidylcholine-induced depolarisation of cat ventricular myocardium. Circ.Res. 52, 543-556.

Clusin W.T., Buchbinder M. and Harrison D.C. (1983). Calcium overload, injury current, and early ischaemic cardiac arrhythmias - a direct connection. Lancet (5 February 1983), p 272.

Coker S.J. (1984). Further evidence that thromboxane exacerbates arrhythmias: Effects of UK 38485 during coronary artery occlusion and reperfusion in aneasthetized greyhounds. J.Mol.Cell.Cardiol. 16, 633-641.

Coker S.J. and Parratt J.R. (1984). The effects of nafazatrom on arrhythmias and prostanoid release during coronary artery occlusion and reperfusion in anaesthetized greyhounds. J.Mol.Cell.Cardiol. 16, 43-52.

Coker S.J., Parratt J.R., Ledingham I.Mc.A. and Zeitlin I.J. (1981). Thromboxane and prostacyclin release from ischaemic myocardium in relation to arrhythmias. Nature. 291, 323-324.

Corr P.B., Ahumada G.G. and Sobel B.E. (1982). Membrane active metabolites: Potential mediators of sudden cardiac death. In: Vogel J.H.K. ed. Cardiovascular medicine vol 1, pp 161-175. New York: Raven Press.

Corr P.B., Cain M.E., Witkowski F.X., Price D.A. and Sobel B.E. (1979). Potential arrhythmogenic electrophysiological derangements in canine Purkinje fibers induced by lysophosphoglycerides. Circ.Res. 44, 822-832.

Corr P.B. and Sharma A.D. (1984). α- adrenergic mediated effects on myocardial calcium. In: Opie L.H. ed. Calcium antagonists and cardiovascular disease, pp 193-204. New York: Raven Press.

Corr P.B. Sharma A.D. and Sobel B.E. (1983). Temporal changes in lysophosphatides in ischaemic myocardium and relation to the severity of dysrhythmia. Circulation. 68, supp. III, 1559.

Corr P.B., Shayman J.A., Kramer J.B. and Kipnis R.J. (1981). Increased α- adrenergic receptors in ischaemic cat myocardium. A potential mediator of electrophysiological derangements. J.Clin.Invest. 67, 1232-1236.

Corr P.B., Snyder D.W., Cain M.E., Crafford W.A., Gross R.W. and Sobel B.E. (1981). Electrophysiological effects of amphiphiles on canine Purkinje fibers: Implications for dysrhythmia secondary to ischaemia. Circ.Res. 49, 354-363.

Corr P.B., Snyder D.W., Lee B.I., Gross R.W., Keim C.R. and Sobel B.E. (1982). Pathophysiological concentrations of lysophosphatides and the slow response. Am.J.Physiol. 243, H187-H195.

Corr P.B. and Sobel B.E. (1979). The importance of metabolites in the genesis of ventricular dysrhythmia induced by ischaemia. Mod.Concepts. Cardiovasc.Dis. 48, 43-47.

Corr P.B. and Sobel B.E. (1980). The role of biochemical factors in ventricular dysrhythmias accompanying ischaemia. Adv.Cardiol. 27, 346-360.

Corr P.B. and Sobel B.E. (1981). Amphiphilic lipid metabolism and ventricular arrhythmias. In: Parratt J.R. ed. Early ischaemic induced arrhythmias. London: Livingstone Churchill.

Corr P.B., Witkowski F.X. and Sobel B.E. (1978). Mechanisms contributing to malignant dysrhythmias induced by ischaemia in the cat. J.Clin.Invest. 61, 109-119.

Cranefield P.F. (1977). Action potentials, after potentials and arrhythmias. Circ.Res. 41, 416-423.

Dangman K.H. and Hoffman B.F. (1983). Antiarrhythmic effects of ethmozin in cardiac Purkinje fibers: Suppression of automaticity and abolition of triggering. J.Pharmacol.Exp.Ther. 227, 578-586.

Daugherty A., Frayn K.N., Redfern W.S. and Woodward B. (1986). The role of catecholamines in the production

of ischaemia induced ventricular arrhythmias in the rat *in vivo* and *in vitro*. Br.J.Pharmac. 87, 265-277.

Davey M.J. (1980). Relevant features of the pharmacology of prazosin. J.Cardiovasc.Pharmacol. 2, 5287-5301.

Davies M.J.,Woolf N. and Robertson W.B. (1976). Pathology of acute myocardial infarction with particular reference to occlusive coronary thrombi. Br.Heart.J. 38, 659-664.

Deka N., Sun G.Y., MacQuarrie R. (1986). Purification and properties of acyl-CoA :1-acyl-SN-glycero-3-phosphocholine-O-acyltransferase from bovine brain microsomes. Arch.Biochem.Biophys. 246, 554-563.

DeMello W.C. (1975). Effect of intracellular injection of calcium and strontium on cell communication in heart. J.Physiol. 250, 231-245.

DeMello W.C. (1982). Intercellular communication in cardiac muscle. Circ.Res. 51, 2-9.

DeMello W.C., Motta G.E. and Chapeau M. (1969). A study on the healing over of myocardial cells of toads. Circ.Res. 24, 475-487.

DeWinter J.M., Korpancova J. and van den Bosch H. (1984). Regulatory aspects of rat liver mitochondrial phospholipase A_2. Effects of calcium ions and calmodulin. Arch.Biochem.Biophys. 234, 243-252.

Dise C.A., Burch J.W. and Goodman D.B.P. (1982). Direct interaction of mepacrine with erythrocyte and platelet membrane phospholipid. J.Biol.Chem. 257,

4701-4704.

Dittmer J.C. and Lester R.L. (1964). A simple specific spray for the detection of phospholipids on thin layer chromatography. J.Lipid.Res. 5, 126-127.

Dobson J.G. and Mayer S.E. (1973). Mechanisms of activation of cardiac glycogen phosphorylase in ischaemia and anoxia. Circ.Res. 33, 412-420.

Douglas C.E., Chan A.C. and Choy P.C. (1986). Vitamin E inhibits platelet phospholipase A_2. Biochim.Biophys.Acta. 876, 639-646.

Downar E., Janse M.J. and Durrar D. (1977a). The effect of coronary artery occlusion on subepicardial transmembrane potentials in the intact porcine heart. Circulation. 56, 217-224.

Downar E, Janse M.J. and Durrar D. (1977b). The effect of 'ischaemic' blood on transmembrane potentials of normal porcine ventricular myocardium. Circulation. 55, 455-462.

Edgar A.D., Strosznajder J. and Horrocks L.A. (1982). Activation of ethanolamine phospholipase A_2 in brain during ischaemia. J.Neurochem. 39, 1111-1116.

Eisner D.A., Allen P.G. and Orchard C.H. (1985). Regulation of resting calcium concentration in cardiac muscle. In: Parratt J.R. ed. Control and manipulation of calcium movement, pp 65-87. New York Raven Press.

Elharrar V., Foster P.R., Jirak T.L., Gaum W.E. and Zipes D.P. (1977). Alterations in canine myocardial excitability during ischaemia. Circ.Res. 40, 98-105.

Ellis E.F., Wright K.F., Wei E.P. and Kontos H.A. (1981). Cyclooxygenase products of arachidonic acid metabolism in cat cerebral cortex after experimental concussive brain injury. J.Neurochem. 37, 892-896.

El-Maraghi N.R.H. and Sealey B.J. (1980). Recurrent myocardial infarction in a young man due to coronary arterial spasm demonstrated at autopsy. Circulation. 61, 199-207.

Engel H.J., Page H.L. and Campbell W.B. (1976). Coronary artery spasm as the cause of myocardial infarction during coronary angiography. Am.Heart.J. 91, 501-506.

Etienne J. and Polonovski J. (1984). Phospholipase A_2 activity in rat and human lymphocytes. Biochem.Biophys.Res.Comm. 125, 719-727.

Evans J.R., Opie L.H. and Shipp J.C. (1963). Metabolism of palmitic acid in perfused rat heart. Am.J.Physiol. 205, 766-770.

Fink K.L. and Gross R.W. (1984). Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds. Circ.Res. 55, 585-594.

Finkelstein S.D., Gilfor D. and Farber J.C. (1985). Alterations in the metabolism of lipids in ischaemia of the liver and kidney. J.Lipid.Res. 26, 726-734.

Fiske C.H. and Subbarow Y. (1925). The colorimetric determination of phosphorus. J.Biol.Chem. 66, 375-400.

Fleischer R.S., Klouwen H. and Brierley G. (1961). Studies of the electron transfer system XXXVIII. Lipid composition of purified enzyme preparations derived from beef heart mitochondria. J.Biol.Chem. 236, 2936-2941.

Flower R.J. (1978). Steroidal anti-inflammatory drugs as inhibitors of phospholipase A_2. Adv.Prostaglandin and Thromboxane Res. 3, 105-112.

Flower R.J. and Blackwell G.J. (1976). The importance of phospholipase A_2 in prostaglandin biosynthesis. Biochem.Pharmac. 25, 285-291.

Folch J., Lees M. and Sloane-Stanley G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissue. J.Biol.Chem. 226, 497-509.

Franson R.C., Pang D.C., Towle D.W. and Weglicki W.B. (1978). Phospholipase A activity of highly enriched preparations of cardiac sarcolemma from hamster and dog. J.Mol.Cell.Cardiol. 10, 921-930.

Garlick P.B., Radda G.K. and Seeley P.J. (1979). Studies of acidosis in the ischaemic heart by phosphorus nuclear magnetic resonance. Biochem.J. 184, 547-554.

Gaudel Y. and Duvelleroy M.A. (1984). Role of oxygen radicals in cardiac injury due to reoxygenation.

J.Mol.Cell.Cardiol. 16, 459-470.

Glaser K.B. and Jacobs R.S. (1986). Molecular pharmacology of manoalide. Inactivation of bee venom phospholipase A_2. Biochem.Pharmac. 35, 449-453.

Gloster J.and Harris P. (1969). The lipid composition of mitochondrial and microsomal fractions of human myocardial homogenates. Cardiovasc.Res. 3, 45-51.

Gloster J. and Harris P. (1970). The lipid composition of mitochondrial and microsomal fractions of rat myocardial homogenates. Cardiovasc.Res. 4, 1-5.

Goldstein I.M. and Weissman G. (1977). Effects of the generation of superoxide anion on permeability of liposomes. Biochem.Biophys.Res.Comm. 75, 604-609.

Gorlin R. and Herman M.V. (1978). Physiology of the coronary circulation. In: Hurst J.W. ed. The heart: arteries and veins, pp 101-106. McGraw-Hill.

Green F.J. and Watanabe A.M. (1985). Cellular mechanisms of action of calcium channel antagonists. In: Parratt J.R. ed. Control and manipulation of calcium movement. New York, Raven Press.

Gross R.W., Corr P.B., Lee B.I., Saffitz J.E., Crafford W.A. and Sobel B.E. (1982). Incorporation of radiolabelled lysophosphatidylcholine into canine purkinje fibers and ventricular muscle. Circ.Res. 51, 27-36.

Gross R.W., Drisdel R.C. and Sobel B.E. (1983). Rabbit myocardial lyosphospholipase transacylase.

J.Biol.Chem. 258, 15165-15172.

Gross R.W. and Sobel B.E. (1980). Isocratic high performance liquid chromatography separation of phosphoglycerides and lysophosphoglycerides. J.Chromatog. 197, 79-85.

Gross R.W. and Sobel B.E. (1981a). Inhibition of myocardial lysophosphatidylcholinetransacylase by palmitoylcarnitine: Implications for arrhythmogenesis. Trans.Assoc.Am.Phys. 94, 115-125.

Gross R.W. and Sobel B.E. (1981b). Inhibition of myocardial lysophosphatidylcholine (LPC) acyl transferase by glycerophosphorylcholine. Clin.Res. 29, 201A.

Gross R.W. and Sobel B.E. (1982). Lysophosphatidylcholine metabolism in the rabbit heart. Characterisation of metabolic pathways and partial purification of myocardial lysophospholipase-transacylase. J.Biol. Chem. 257, 6702-6708.

Gross R.W. and Sobel B.E. (1983). Rabbit myocardial cytosolic lysophospholipase. Purification, characterisation and competitive inhibition byL-α palmitoylcarnitine. J.Biol.Chem. 258, 5221-5226.

Gruetter C.A., Gruetter D.Y., Lyon J.E., Kadowitz P.J. and Ignarro L.J. (1981). Relationship between cyclic GMP formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide. J.Pharmac.Exp.Ther. 219,

181-186.

Harrison M.J.G., Pollock S.S., Steiner M. and Weisblatt E. (1985). The effect of red blood cells on platelet behaviour- a study of whole blood aggregation. Int.Congr.Ser-Excerpta.Med. 687, 201-205.

Hearse D.J., Humphrey S.M. and Chain E.B. (1973). Abrupt reoxygenation of the anoxic potassium-arrested rat heart: A study of myocardial enzyme release. J.Mol.Cell.Cardiol. 5, 395-407.

Hearse D.J., Humphrey S.M., Nayler W.G. Slade A and Border D. (1975). Ultrastructural damage associated with reoxygenation of the anoxic myocardium. J.Mol.Cell.Cardiol. 7, 315-324.

Helenius A. and Simons K. (1975). Solubilisation of membranes by detergents. Biochim.Biophys.Acta. 415, 29-79.

Herrick J.B. (1912). Clinical features of sudden obstruction of the coronary artery. J.Am.Med.Assoc. 59, 2015-2020.

Hess H.H. and Derr J.E. (1975). Assay of inorganic and organic phosphorus in the 0.1-5 nmole range. Anal.Biochem. 63, 607-613.

Hill J.L. and Gettes L.S. (1980). Effect of acute coronary artery occlusion on local myocardial extracellular K^+ activity in swine. Circulation. 61, 768-778.

Hillis L.D. and Braunwald E. (1977). Myocardial ischaemia. New.Eng.J.Med. 296, 971-1096.

Hirche H., Franz C., Bös L., Bissig R., Lang R. and Schramm M. (1980). Myocardial extracellular K^+ and H^+ increase and noradrenaline release as possible cause of early arrhythmias following acute coronary artery occlusion in pigs. J.Mol.Cell.Cardiol. 12, 579-593.

Hoerter J.A., Miceli M.V., Renlund D.G., Jacobus W.E., Gerstenblith G. and Lakatta E.G. (1986). A phosphorus 31 nuclear magnetic resonance study of the metabolic, contractile, and ionic consequences of induced calcium alterations in the isovolumic rat heart. Circ.Res. 58, 539-551.

Hoffstein S., Weissman G. and Fox A.C. (1976). Lysosomes in myocardial infarction: Studies by means of cytochemistry and subcellular fractionation, with observations on the effects of methylprednisolone. Circulation. 53, supp I, 34-40.

Hofmann S.L., Prescott S.M. and Majerus P.W. (1982). The effects of mepacrine and p-bromophenacyl bromide on arachidonic acid release in human platelets. Arch.Biochem.Biophys. 215, 237-244.

Hostetler K.Y. and Matsuzawa Y. (1981). Studies on the mechanism of drug induced lipidosis. Cationic amphiphilic drug inhibition of lysosomal phospholipases A and C. Biochem.Pharmac. 30, 1121-

1126.

Hseuh W, Isakson P.C. and Needleman P. (1977). Hormone selective lipase activation in the isolated rabbit heart. Prostaglandins 13, 1073-1091.

Hülsmann W.C., Stam H. and Lamers J.M.J. (1985). Localisation and function of lipases and their reaction products in the rat heart. In: Ferrari R., Katz A.M., Shug A. and Visioli O., ed. Myocardial ischaemia and lipid metabolism, pp 27-37.

Idell-Wenger J.A., Grotyohann L.W. and Neely J.R. (1978). Coenzyme A and carnitine distribution in normal and ischaemic hearts. J.Biol.Chem. 253, 4310-4318.

Jacob M.I. and Berne R.M. (1960). Metabolism of purine derivatives by the isolated cat heart. Am.J.Physiol. 198, 322-326.

Janse M.J., Kléber A.G., Capucci A., Coronel R. and Wilms-Schopman F. (1986). Electrophysiological basis for arrhythmias caused by acute ischaemia: Role of the subendocardium. J.Mol.Cell.Cardiol. 18,339-355.

Jennings R.B. (1970). Myocardial ischaemia: Observations, definitions and speculations. J.Mol.Cell.Cardiol. 1, 345-349.

Jennings R.B. and Ganote C.E. (1974). Structural changes in myocardium during acute ischaemia. Circ.Res. 35, supp III 156-168.

Jennings R.B., Reimer K.A., Hill M.L. and Mayer S.E. (1981). Total ischaemia in dog hearts in vitro. 1.

Comparison of high energy phosphate production, utilisation and depletion and of adenine nucleotide catabolism in total ischaemia *in vitro* vs severe ischaemia *in vivo*. Circ.Res. **49**, 892-900.

Jennings R.B., Reimer K.A. and Steenbergen C. (1985). Myocardial ischaemia and reperfusion. Role of calcium. In: Parratt J.R. ed. Control and manipulation of calcium movement, pp 273-302. New York, Raven Press.

Johnson A.J. and MacCarty W.R. (1959). The lysis of artificially induced intravascular clots in man by i.v. infusion of streptokinase. J.Clin.Invest. **38**, 1627-1643.

Jungalwala F.B., Evans J.E. and McCluer R.H. (1976). High performance liquid chromatography of phosphatidylcholine and sphingomyelin with detection in the region of 200nm. Biochem.J. **155**, 55-60.

Jungalwala F.B., Turel R.J., Evans J.E. and McCluer R.H. (1975). Sensitive analysis of ethanolamine and serine containing phosphoglycerides by high performance liquid chromatography. Biochem.J. **145**, 517-526.

Kalsner S. (1982). Vasoconstrictors, spasm and acute myocardial events. In: Kalsner S. ed. The coronary artery, pp 551-595. London: Croom-Helm Ltd.

Kane K.A., Morcillo-Sanchez E.J., Parratt J.R., Rodger I.W. and Shahid M. (1985). The relationship between

coronary artery occlusion-induced arrhythmias and myocardial cyclic nucleotide levels in the anaesthetized rat. Br.J.Pharmac. 84, 139-145.

Kannengiesser G.J., Lubbe W.F. and Opie L.H. (1975). Experimental myocardial infarction with left ventricular failure in the isolated perfused rat heart: Effects of isoproterenol and pacing. J.Mol.Cell.Cardiol. 7, 135-151.

Kaplinsky E., Ogawa S., Balke C.W. and Dreifus L.S. (1979). Two periods of early ventricular arrhythmias in the canine acute infarction model. Circulation 60, 397-403.

Kärki N.T. (1958). The effects of changes in ion concentration on ventricular fibrillation induced electrically. J.Physiol. 141, 366-376.

Karli J.N., Karikas G.A., Hatzipavlou P.K., Levis G.M. and Moulopoulos S.N. (1979). The inhibition of Na^+ and K^+ stimulated ATPase activity of rabbit and dog heart sarcolemma by lysophosphatidylcholine. Life.Sci. 24 , 1869-1876.

Katz A.M. (1982). Membrane derived lipids and the pathogenesis of ischaemic myocardial damage. J.Mol.Cell.Cardiol. 14, 627-632.

Katz A.M. and Reuter H. (1979). Cellular calcium and cardiac cell death. Am.J.Cardiol. 44, 188-190.

Kennedy J.W., Ritchie J.L., Davies K.B., Stadius M.L., Maynard C. and Fritz J.K. (1985). The Western

Washington randomised trial of intracoronary streptokinase in acute myocardial infarction. New.Eng.J.Med. 312, 1073-1078.

Kihlstrom M. and Salminen A. (1985). Inhibition of lipid peroxidation of cardiac muscle in vitro by phospholipases and surfactants. J.Mol.Cell.Cardiol. 17, supp 3, 148.

Klabunde R.E. (1983). Dipyridamole inhibition of adenosine metabolism in human blood. Eur.J.Pharmacol. 93, 21-26.

Kleber A.G. (1983). Resting membrane potential, extracellular activity and intracellular sodium activity during acute global ischaemia in isolated perfused guinea pig hearts. Circ.Res. 52, 442-450.

Kléber A.G. (1984). Extracellular potassium accumulation in acute myocardial ischaemia. J.Mol.Cell.Cardiol. 16, 389-394.

Klein I., Snowdowne K. and Borle A. (1982). Studies of intracellular free calcium in isolated heart cells. J.Cell.Biol. 95, 376a.

Klopfenstein W.E. (1969). Enthalpy change of binding lysolecithin to serum albumin. Biochim.Biophys.Acta. 181, 323-332.

Knabb M.T., Saffitz J.E., Corr P.B. and Sobel B.E. (1986). The dependence of electrophysiological derangements on accumulation of endogenous long chain acyl carnitine in hypoxic neonatal rat

myocytes. Circ.Res. 58, 230-240.

Kontos H.A., Wei E.P., Dietrich W.D., Navari R.M., Povlishock J.T., Ghatak N.R., Ellis E.F. and Patterson J.L. (1981). Mechanism of cerebral arteriolar abnormalities after acute hypertension. Am.J.Physiol. 240, H511-H527.

Korner C.F., Hausmann G. Gemsa D and Resch K. (1984). Rate of prostaglandin synthesis is not controlled by phospholipase A activity but by reincorporation of released fatty acids into phospholipids. Agents and Actions 15, 29-31.

Kramer J.B., Davies A.G., Dean P., McCluskey E.R., Needleman P, and Corr P.B. (1985). Thromboxane A_2 does not contribute to arrhythmogenesis during evolving canine myocardial infarction. J.Cardiovasc.Pharmac. 7, 1069-1076.

Kruger F.A. , Leighty E.G. and Weissler A.M. (1967). Catecholamine stimulation of myocardial lipolysis and fatty acid reesterification. J.Clin.Invest. 46, 1080-1081.

Kunze H., Nahas N., Traynor J.R. and Wurl M. (1976). Effects of local anaesthetics on phospholipases. Biochem.Biophys.Acta. 441, 93-102.

Kurien V.A. and Oliver M.F. (1970). Metabolic cause for arrhythmias during acute myocardial hypoxia. Lancet ii) 813-815.

Kurien V.A., Yates P.A. and Oliver M.F. (1971). The role of free fatty acids on the production of ventricular arrhythmias after acute coronary artery occlusion. Eur.J.Clin.Invest. 1, 225-241.

Laine G.A. and Granger H.J. (1985). Microvascular, interstitial and lymphatic interactions in normal heart. Am.J.Physiol. 249, H834-H842.

Langer G.A. (1985). The effect of pH on cellular and membrane calcium binding and contraction of myocardium. Circ.Res. 57, 374-382.

Langer G.A., Frank J.S., Nudd L.M. and Seraydarian K. (1976). Sialic acid : Effect of removal on calcium exchangability of cultured heart cells. Science. 193, 1013-1015.

Lawrence A.J. (1975). Lysolecithin inhibits an action of bee venom phospholipase A_2 in erythrocyte membrane. FEBS Letters. 58, 186-189.

Lawrence A.J., Moores G.R. and Steele J. (1974). A conductimetric study of erythrocyte lysis by lysolecithin and linoleic acid. Eur.J.Biochem. 48, 277-286.

Lee T.W., Ting A. and Vidaver G.A. (1986). Lysolecithin induced Ca^{2+} uptake by pigeon red cells. Life Sci. 38, 1013-1019.

Lehninger A.L. (1975). Biochemistry 2nd edn. New York: Worth Publishers.

Liedtke A.J., Hughes H.C. and Neely J.R. (1976). Effects of coronary perfusion during myocardial hypoxia: comparison of metabolic and haemodynamic events with global ischaemia and hypoxaemia. J.Thorac.Cardiovasc. Surg. 71, 726-735.

Liedtke A.J., Nellis S and Neely J.R. (1978). Effects of excess free fatty acids on mechanical and metabolic function in normal and ischaemic myocardium in swine. Circ.Res. 43, 652-661.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193, 265-275.

Lubbe W.F., Podzuweit T., Daries P.S. and Opie L.H. (1978). The role of cyclic adenosine monophosphate in adrenergic effects on ventricular vulnerability to fibrillation in the isolated perfused rat heart. J.Clin.Invest. 61, 1260-1269.

Lucas S.K., Gardner T.J., Flaherty J.T., Bulkley B.H., Elmer E.B. and Gott V.L. (1980). Beneficial effects of mannitol administration during reperfusion after ischaemic arrest. Circulation. 62, supp I, 34-41.

MacAlpin R.N. (1974). Fugitive coronary occlusion. New.Eng.J.Med. 291, 470-471.

McCord J.M. (1985). Oxygen derived free radicals in post ischaemic tissue injury. New.Eng.J.Med. 312, 159-163.

Mak I.T., Kramer J.H. and Weglicki W.B. (1986). Potentiation of free radical induced lipid peroxidative injury to sarcolemmal membranes by lipid amphiphiles. J.Biol.Chem. 261, 1153-1157.

Man R.Y.K. and Choy P.C. (1982). Lysophosphatidylcholine causes cardiac arrhythmias. J.Mol.Cell.Cardiol. 14, 173-175.

Man R.Y.K. and Lederman C.L. (1985). Effect of reduced calcium on lysophosphatidylcholine induced cardiac arrhythmias. Pharmacology. 31, 11-16.

Man R.Y.K., Slater T.L., Pelletier M.P. and Choy P.C. (1983). Alterations of phospholipids in ischaemic canine myocardium during acute arrhythmia. Lipids. 18, 677-681.

Man R.Y.K., Wong T. and Choy P.C. (1983). Effects of lysophosphoglycerides in cardiac arrhythmias. Life Sci. 32, 1325-1330.

Manning A.S. and Hearse D.J. (1984). Reperfusion induced arrhythmias: Mechanisms and prevention. J.Mol.Cell.Cardiol. 16, 497-518.

Martonosi A., Donley J. and Halpin R. (1968). Sarcoplasmic reticulum III. The role of phospholipids in adenosine triphosphatase activity and Ca^{2+} transport. J.Biol.Chem. 243, 61-70.

Martonosi A., Donley J., Purcell A.C. and Halpin R.A. (1971). Sarcoplasmic reticulum XI. The mode of involvement of phospholipids in the hydrolysis of

ATP by sarcoplasmic reticulum membranes. Arch.Biochem.Biophys. 144, 529-540.

Meerson F.Z., Kagan V.E., Kozlov Y.P., Belkina L.M. and Arkhipenko Y.V. (1982). The role of lipid peroxidation in pathogenesis of ischaemic damage and the antioxidant protection of the heart. Basic.Res.Cardiol. 77, 465-485.

Miura Y., Inui J. and Imamura H. (1978). Alpha-adrenoceptor mediated restoration of calcium dependent potential in the partially depolarised rabbit papillary muscle. Nauyn-Schmiedebergs Arch.Pharmacol. 301, 201-205.

Miwa M., Ichihashi T., Motojima H., Onodera-Kubota I. and Matsumoto M. (1985). Studies on phospholipase A inhibitor in blood plasma II. Interaction of phospholipase A inhibitor with phospholipase A and its specificity. J.Biochem. 98, 157-165.

Mogelson S. Wilson G.E. and Sobel B.E. (1980). Characterisation of rabbit myocardial phospholipids with P^{31} nuclear magnetic resonance. Biochim. Biophys. Acta. 619, 680-688.

Moncada S. and Vane J.R. (1978). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. Pharmacol.Rev. 30, 293-331.

Moore K.H., Radlorf J.F., Hull F.E. and Sweeley C.C. (1980). Incomplete fatty acid oxidation by ischaemic

heart: β hydroxy fatty acid production. Am.J.Physiol. 239, H257-H265.

Morena H., Janse M.J., Fiolet J.W.T., Krieger W.J.G., Crijns H. and Durrar D. (1980). Comparison of the effects of regional ischaemia, hypoxia, hyperkalemia and acidosis on intracellular and extracellular potentials and metabolism in the isolated porcine heart. Circ.Res. 46, 634-646.

Moskowitz N., Andres A., Silva W., Shapiro L., Schook W. and Puszkin S. (1985). Calcium dependent binding of calmodulin to phospholipase A_2 subunits induces enzymatic activation. Arch.Biochem.Biophys. 241, 413-417.

Moskowitz N., Shapiro L., Schook W. and Puszkin S. (1983). Phospholipase A_2 modulation by calmodulin, prostaglandins and cyclic nucleotides. Biochem.Biophys.Res.Comm. 115, 94-99.

Mowbray J., Bates D.J. and Perrett D. (1981). Inversely related oscillations in the contents of cGMP and the total adenine nucleotides in steady state perfused rat hearts. FEBS Letters. 131, 55-59.

Nakaya H., Kimura S. and Kanno M. (1984). Suppression of lysophosphatidylcholine induced abnormal automaticity by verapamil in canine Purkinje fibers. Jpn.J.Pharmacol. 36, 371-378.

Nakaya H., Ozaki S., Kimura S., Gotoh Y. and Kanno M. (1984). Effects of verapamil on electrophysiological

disturbances induced by lysophosphatidylcholine in isolated rabbit hearts. Arch.Int.Pharmacodyn. 270, 88-96.

Nalbone G. and Hostetler K.Y. (1985). Subcellular localisation of the phospholipases A ofrat heart: evidence for a cytosolic phospholipase A_1. J.Lipid.Res. 26, 104-114.

Nathan R.D. and Bhattacharyya M.L. (1981). Membrane sialic acid and electrophysiology of cardiac Purkinje fibres. Biophys.J. 33, 34a.

Needleman P., Wyche A., Sprecher H., Elliott W.J. and Evers A. (1985). A unique cardiac cytosolic acyltransferase with preferential selectivity for fatty acids that form cyclooxygenase/lipoxygenase metabolites and reverse essential fatty acid deficiency. Biochim.Biophys.Acta. 836, 267-273.

Neely J.R. and Morgan H.E. (1974). Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. Ann.Rev.Physiol. 36, 413-459.

Neely J.R., Rovetto M.J. and Oram J.F. (1972). Myocardial utilisation of carbohydrate and lipids. Prog.Cardiovasc.Dis. 15, 289-329.

Newsholme E.A. (1971/72). The regulation of phosphofructokinase in muscle. Cardiology 56, 22-24.

Noma A. (1983). ATP regulated K^+ channels in cardiac muscle. Nature 305, 147-148.

Noronha-Dutra A.A. and Steen E.M. (1982). Lipid peroxidation as a mechanism of injury in cardiac myocytes. Lab.Invest. 47, 346-353.

Oliva P.B., Potts D.E. and Pluss R.G. (1973). Coronary arterial spasm in Prinzmetal angina: Documentation by coronary arteriography. New.Eng.J.Med. 288, 745-751.

Oliver M.F., Kurien V.A. and Greenwood T.W. (1968). Relation between serum fatty acids and arrhythmia and death after acute myocardial infarction. Lancet 1, 710-715.

Olson H.G., Butman S.M., Piters K.M., Gardin J.M., Lyons K.P., Jones L., Chilazi G.,Kumar K.L.A. and Colombo A. (1986). A randomized controlled trial of intravenous streptokinase in evolving acute myocardial infaction. Am.Heart.J. 111, 1021-1029.

Op Den Kamp J.A.F., Roelofsen B. and van Deenen L.L.M. (1985). Structural and dynamic aspects of phosphatidylcholine in the human erythrocyte membrane. TIBS. 10, 320-323.

Opie L.H. (1968). Metabolism of the heart in health and disease. Am.Heart.J. 76, 685-698.

Opie L.H., Norris R.N., Thomas M., Holland A.J., Owen P. and Van Noorden S. (1971). Failure of high concentrations of circulating free fatty acids to

provoke arrhythmias in experimental myocardial infarction. Lancet i, 818-822.

Osterreider W., Brum G., Hescheler J., Trautwein W., Flockerei V. and Hofmann F. (1982). Injection of subunits of cyclic AMP dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. Nature. 298, 576-578.

Pang D.C. and Weglicki W.B. (1977). Cardiac sarcolemma of the hamster. Enrichment of the (Na/K) ATPase. Biochim.Biophys.Acta. 465, 411-414.

Philipson K.D., Bers D.M. and Nishimoto A.Y. (1980). The role of phospholipids in the calcium binding of isolated cardiac sarcolemma. J.Mol.Cell.Cardiol. 12, 1159-1173.

Podrid P.J. (1985). Can antiarrhythmic drugs prevent sudden death? In: Reiser H. and Horowitz L. eds. Mechanisms and treatment of cardiac arrhythmias: Relevance of basic studies to clinical management, pp 341-361. Urban and Schwarzenberg.

Podzuweit T. (1982). Cyclic AMP arrhythmias. Inhibition by choline esters. Adv.Myocardiol. 3, 193-198.

Pogwizd S.M., Sharma A.D. and Corr P.B. (1982). Influences of labetolol, a combined α and β blocking agent on the dysrhythmias induced by coronary occlusion and reperfusion. Cardiovasc.Res. 16, 398-407.

Poole-Wilson P.A., Harding D.P., Bourdillon P.D.V. and Tones M.A. (1984). Calcium out of control. J.Mol.Cell.Cardiol. 16, 175-187.

Prakash R., Parmley W.W., Horvat M. and Swan H.J.C. (1972). Serum cortisol, plasma free fatty acids, and urinary catecholamines as indicators of complications in acute myocardial infarction. Circulation 45, 736-745.

Ransnäs L., Gjörstrup P., Hjalmarson A., Sjögren C-G. and Jacobsson B. (1986). Muscarinic receptors in mammalian myocardium: Effects of atrial and ventricular receptors on phosphatidylinositol metabolism and adenylate cyclase. J.Mol.Cell.Cardiol. 18, 807-814.

Rapundalo S.T., Briggs F.N. and Feher J.J. (1986). Effects of ischaemia on the isolation and function of canine cardiac sarcoplasmic reticulum. J.Mol.Cell.Cardiol. 18, 837-851.

Rau E.E., Shine K.I. and Langer G.A. (1977). Potassium exchange and mechanical performance in anoxic mammalian myocardium. Am.J.Physiol. 232, H85-H94.

Read S.M. and Northcote D.H. (1981). Minimisation of variation in the response to different proteins of the Coomassie Blue G dye binding assay for protein. Anal.Biochem. 116, 53-64.

Reichenbach D.D., Moss N.S. and Meyer E.M. (1977). Pathology of the heart in sudden death.

Am.J.Cardiol. 39, 865-872.

Reimer K.A. and Jennings R.B. (1979). The "wavefront phenomenon" of myocardial ischaemic cell death. II Transmural progression of necrosis within the framework of ischaemic bed size (myocardium at risk) and collateral flow. Lab.Invest. 40, 633-644.

Richards D.E., Irvine R.F. and Dawson R.M.C. (1979). Hydrolysis of membrane phospholipids by phospholipases of rat liver lysosomes. Biochem.J. 182, 599-606.

Ridolfi R.L. and Hutchins G.M. (1977). The relationship between coronary artery lesions and myocardial infarcts: Ulceration of atherosclerotic plaques precipitating coronary thrombosis. Am.Heart.J. 93, 468-486.

Riemersma R. and Michorowski B.(1983). Lysolecithin and acute coronary ligation in the anaesthetized dog. Cardiovasc.Res. 17, 505-508.

Roberts W.C. and Buja L.M. (1972). The frequency and significance of coronary arterial thrombi and other observations in fatal acute myocardial infarction. Am.J.Med. 52, 425-443.

Rothman J.E. and Lenard J. (1977). Membrane asymmetry. Science 195, 743-753.

Rovetto M.J., Lamberton W.F. and Neely J.R. (1975). Mechanisms of glycolytic inhibition in ischaemic rat hearts. Circ.Res. 37, 742-751.

Russell D.C., Oliver M.F. and Wojyczak J. (1977). Combined electrophysiological technique for assessment of the cellular basis of early ventricular arrhythmias. Lancet ii , 686-688.

Sarnama M., Szwarcer E., Conard J. and Horellou M.H. (1985). Thrombolysis. In: Poller L. ed. Recent advances in blood coagulation 4. Churchill Livingstone.

Savard J.D. and Choy P.C. (1982). Phosphatidylcholine formation from exogenous lysophosphatidylcholine in isolated hamster heart. Biochim.Biophys.Acta. 711, 40-48.

Sawicki G.J. and Arnsdorf M.F. (1985). Electrophysiologic actions and interactions between lysophosphatidylcholine and lidocaine in the non steady state: The match between multiphasic arrhythmogenic mechanisms and multiple drug effects in cardiac Purkinje fibers. J.Pharm.Exp.Ther. 235, 829-838.

Schaper J. (1979). Ultrastructure of the myocardium in acute ischaemia. In: Schaper W. ed. The pathophysiology of myocardial perfusion, pp 581-674. Elsevier: Amsterdam.

Schaper W. (1984). Experimental infarcts and the microcirculation. In: Hearse D.J. and Yellon D.M. eds. Therapeutic approaches to myocardial infarct size limitation, pp 79-90. New York: Raven Press.

Schrader J., Haddy F.J. and Gerlach E. (1977). Release of adenosine, inosine and hypoxanthine from the isolated guinea pig heart during hypoxia, flow autoregulation and reactive hyperaemia. Pluegers.Arch. 369, 1-6.

Schwartz C.J., Chandler A.B., Gerrity R.G. and Naito H.K. (1978). Clinical and pathological aspects of arterial thrombosis and thromboembolism. Adv.Exp.Med.Biol. 104, 111-126.

Sedlis S.P., Corr P.B., Sobel B.E. and Ahumada G.G. (1983). Lysophosphatidylcholine potentiates Ca^{2+} accumulation in rat cardiac myocytes. Am.J.Physiol. 244, H32-H38.

Sevanian A., Stein R.A. and Mead J.F. (1981). Metabolism of epoxidised phosphatidylcholine by phospholipase A_2 and epoxide hydrolase. Lipids.16, 781-789.

Shaikh N.A. and Downar E. (1980). Assessment of various techniques for the quantitative extraction of lysophospholipids from myocardial tissue. Clin.Res. 28, 211a.

Shaikh N.A. and Downar E. (1981). Time course of changes in porcine myocardial phospholipid levels during ischaemia. A reassessment of the lysolipid hypothesis. Circ.Res. 49, 316-325.

Shaikh N.A. and Downar E. (1985). Activation of phospholipases during myocardial ischaemia and their probable role in arrhythmogenesis.

Dev.Cardiovasc.Med. 49, 298-316.

Shaikh N.A. and Palmer F.B. (1976). Deposition of lipids in the developing central nervous system of the chicken. J.Neurochem. 26, 597-603.

Sharma A.D., Ahumada G.G., Sobel B.E. and Corr P.B. (1983). Lysophosphatide induced increases in α adrenergic receptor number in intact myocytes. Circulation. 68, supp III, 225.

Sheridan D.J., Penkoske P.A., Sobel B.E. and Corr P.B. (1980). Alpha adrenergic contributions to dysrhythmia during myocardial ischaemia and reperfusion. J.Clin.Invest. 65, 161-171.

Sheu S-S and Fozzard H.A. (1982). Transmembrane Na^+ and Ca^{2+} electrochemical gradients in cardiac muscle and their relationship to force development. J.Gen.Physiol. 80, 325-351.

Shlafer M., Kane P.F. and Kirsh M.M. (1982). Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischaemic, perfused heart. J.Thorac.Cardiovasc.Surg. 83, 830-839.

Shug A.L., Thomsen J.H., Folts J.D., Bittar N., Klein M.I., Koke J.R. and Huth P.J. (1978). Changes in tissue levels of carnitine and other metabolites during myocardial ischaemia and anoxia. Arch.Biochem.Biophys. 187, 25-33.

Silver M.D., Baroldi G. and Mariani F. (1980). The relationship between acute occlusive coronary thrombi and myocardial infarction studied in 100 consecutive patients. Circulation. 61, 219-227.

Simon M-F., Chap H. and Douste-Blazy L. (1986). Selective inhibition of human platelet phospholipase A_2 by buffering cytoplasmic calcium with the fluorescent indicator quin 2. Evidence for different calcium sensitivities of phospholipases A_2 and C. Biochim.Biophys.Acta. 875, 157-164.

Smith E.F., Lefer A.M. and Smith J.B. (1980). Influence of thromboxane inhibition on the severity of myocardial ischaemia in cats. Can.J. Physiol. Pharmacol. 58, 294-300.

Snowdowne K.W., Ertel R.J. and Borle A.B. (1985). Measurement of cytosolic calcium with aequorin in dispersed rat ventricular cells. J.Mol.Cell.Cardiol. 17, 233-241.

Snyder A.W. (1969). The biochemistry of lipids containing ether bonds. Prog.Chem.Fats.Other Lipids. 10, 287-335.

Snyder D.W., Crafford W.A., Glashow J.L., Rankin D., Sobel B.E. and Corr P.B. (1981). Lysophosphoglycerides in ischaemic myocardium effluents and potentiation of their arrhythmogenic effects. Am.J.Physiol. 241, H700-H707.

Sobel B.E., Corr P.B., Robison A.K., Goldstein R.A., Witkowski F.X. and Klein M.S. (1978). Accumulation of lysophosphoglycerides with arrhythmogenic properties in ischaemic myocardium. J.Clin.Invest. 62, 546-553.

Spath J.A., Lane D.L. and Lefer A.M. (1974). Protective action of methylprednisolone on the myocardium during experimental myocardial ischaemia in the cat. Circ.Res. 35, 44-51.

Srivastava K.J. (1986). Vitamin E exerts antiaggregatory effects without inhibiting the enzymes of the arachidonic acid cascade in platelets. Prost.Leuk.Med. 21, 177-185.

Stallones R.A. (1980). The rise and fall of ischaemic heart disease. Sci.Amer. 243, (5), 43-49.

Stam H. and Hülsman W.C. (1981). Release of lipolytic products from rat heart. Hormonal stimulation, intracardiac origin and pharmacological modification. Biochem.Int. 2, 477-484.

Starnes V.A., Primm R.K., Woosley R.L., Oates J.A. and Hammon J.W. (1982). Administration of prostacyclin prevents ventricular fibrillation following coronary occlusion in conscious dogs. J.Cardiovasc.Pharmac. 4, 765-769.

Staszewska-Barczak J. and Ceremuzynski L. (1968). The continuous estimation of catecholamine release in the early stages of myocardial infarction in the

dog. Clin.Sci. 34, 531-539.

Steenbergen C., Deleeuw G., Barlow C., Chance B. and Williamson J.R. (1977). Heterogeneity of the hypoxic state in perfused rat heart. Circ.Res. 41, 606-615.

Steenbergen C. and Jennings R.B. (1984). Relationship between lysophospholipid accumulation and plasma membrane injury during total *in vitro* ischaemia in dog heart. J.Mol.Cell.Cardiol. 16, 605-621.

Stein Y. and Stein O. (1965). Metabolism of lysolecithin by the perfused rat heart. Biochim.Biophys.Acta. 106, 527-539.

Steinberg D. and Khoo J.C. (1977). Hormone sensitive lipase of adipose tissue. Fed.Proc. 36, 1986-1990.

Stewart J.R., Blackwell W.H., Crute S.L., Loughlin V. Greenfield L.J. and Hess M.L. (1983). Inhibition of surgically induced ischaemia / reperfusion injury by oxygen free radical scavengers. J.Thorac.Surg. 86 262-272.

Storch J. and Schachter D. (1985). Calcium alters the acyl chain composition and lipid fluidity of rat hepatocyte plasma membrane *in vivo*. Biochim.Biophys.Acta. 812, 473-484.

Svingen B.A., Buege J.A., O'Neal F.O. and Aust S.D. (1979). The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. J.Biol.Chem. 254, 5892-5899.

Swoboda G., Fritzsche J. and Hasselbach W. (1979). Effects of phospholipase A_2 and albumin on the calcium dependent ATPase and the lipid composition of sarcoplasmic membranes. Eur.J.Biochem. 95, 77-78.

Takahashi K. and Kako K.J. (1983). The effect of a calcium channel antagonist, Nisoldipine, on the ischaemia induced change of canine sarcolemmal membrane. Basic.Res.Cardiol. 78, 326-337.

Takahashi K. and Kako K.J. (1984). Ischaemia induced changes in sarcolemmal (Na,K) ATPase, K^+-pNPPase, sialic acid and phospholipid in the dog and effects of the nisoldipine and chlorpromazine treatment. Biochem.Med. 31, 271-286.

Thakkar J.K., Sperelakis N., Pang D. and Franson R.C. (1983). Characterisation of phospholipase A_2 activity in rat aorta and smooth muscle cells. Biochim.Biophys.Acta. 750,134-140.

Tibbits G.F., Sasaki M., Ikeda M., Shimada K., Tsuruhara T. and Nagatomo T. (1981). Characterisation of rat myocardial sarcolemma. J.Mol.Cell.Cardiol. 13, 1051-1061.

Tokumura A., Mostafa M.H., Nelson D.R. and Hanahan D.J. (1985). Stimulation of (Ca-Mg) ATPase in human erythrocyte membranes by synthetic lysophosphatidic acids and lysophosphatidylcholines. Effects of chain length and degree of unsaturation of the fatty acid groups. Biochim.Biophys.Acta. 812, 568-574.

Van den Bosch H. (1980). Intracellular phospholipases A. Biochim.Biophys.Acta. 604, 191-246.

Verrier R.L. and Hagestad E.L. (1985). Neural mechanisms in cardiac arrhythmias. In: Reiser H. and Horowitz L. eds. Mechanisms and treatment of cardiac arrhythmias. Relevance of basic studies to clinical management, pp 39-63. Urban and Schwarzenberg.

Victor T., van der Merwe N., Benade A.J.S., LaCock C. and Lochner A. (1985). Mitochondrial phospholipid composition and microviscosity in myocardial ischaemia. Biochim.Biophys.Acta. 834, 215-223.

Vleugels A., Vereecke J. and Carmeleit E. (1980). Ionic currents during hypoxia in voltage clamped cat ventricular muscle. Circ.Res. 47, 501-508.

Waite M. (1985). Approaches to the study of mammalian cellular phospholipases. J.Lipid.Res. 26, 1379-1388.

Warner H.R. and Lands W.E.M. (1961). The metabolism of plasmalogen: Enzymatic hydrolysis of the vinyl ether. J.Biol.Chem. 236, 2402-2409.

Warren L. (1959). The thiobarbituric acid assay of sialic acids. J.Biol.Chem. 234, 1971-1975.

Warren L. (1959a). Thiobarbituric acid assay of sialic acid. Fed.Proc. 18, 347.

Warren G.B., Houslay M.D., Metcalfe J.C. and Birdsall N.J.M. (1975). Cholesterol is excluded from the phospholipid annulus surrounding an active calcium transport protein. Nature. 255, 684-687.

Watanabe A.M. and Besch H.R. (1974). Cyclic adenosine monophosphate modulation of slow calcium influx channels in guinea pig hearts. Circ.Res. 35, 316-324.

Watanabe T.,Hashimoto Y., Teramoto T., Kume S., Naito C. and Oka H. (1986). Calmodulin independent inhibition of platelet phospholipase A_2 by calmodulin antagonists.

Weglicki W.B., Dickens B.F. and Mak I.T. (1984). Enhanced lysosomal phospholipid degradation and lysophospholipid production due to free radicals. Biochim.Biophys.Acta. 124, 229-235.

Weglicki W.B., Kramer J.H., Franson R.C., Pang A.C. and Owens K. (1985). Perturbations of sarcolemma by lipases and amphiphiles. Dev.Cardiovasc.Med. 49, 258-272.

Weglicki W.B., Waite M., Sisson P. and Shohet S.B. (1971). Myocardial phospholipase A of microsomal and mitochondrial fractions. Biochim.Biophys.Acta. 231, 512-519.

Wei E.P., Kontos H.A., Dietrich D., Povishock J.T. and Ellis E.F. (1981). Inhibition by free radical scavengers and by cyclooxygenase inhibitors of pial arteriolar abnormalities from concussive brain injury in cats. Circ.res. 48, 95-103.

Wei E.P., Lamb R.G. and Kontos H.A. (1982). Increased phospholipase C activity after experimental brain

injury. J.Neurosurg. 56, 695-698.

Weltzein H.U. (1979). Cytosolic and membrane perturbing properties of lysophosphatidylcholine. Biochim. Biophys.Acta. 559, 259-287.

Wightman P.D., Dahlgren M.E., Hall J.C., Davies P. and Bonney R.J. (1981). Identification and characterisation of a phospholipase C activity in resident mouse peritoneal macrophages. Biochem.J. 197, 523-526.

Wildenthal K. (1978). Lysosomal alterations in ischaemic myocardium: Result or cause of myocellular damage? J.Mol.Cell.Cardiol. 10, 595-603.

Willebrands A.F., Ter Welle H.F. and Tasserson S.J.A. (1973). The effect of a high molar FFA / albumin ratio in the perfusion medium on rhythm and contractility of the isolated rat heart. J.Mol.Cell.Cardiol. 5, 259-273.

Williams D.A., Fogarty K.E., Tsien R.Y. and Fay F.S. (1985). Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. Nature. 318, 558-561.

Withnall M.T.,Brown T.J. and Diocee B.K. (1984). Calcium regulation of phospholipase A_2 is independent of calmodulin. Biochem.Biophys.Res.Comm. 121, 507-513.

Wolf R.A. and Gross R.W. (1985). Identification of neutral active phospholipase which hydrolyses choline glycerophospholipids and plasmalogen

selective phospholipase A_2 in canine myocardium. J.Biol.Chem. 260, 7295-7303.

Wong P.Y.K. and Cheung W.Y. (1979). Calmodulin stimulates human platelet phospholipase A_2. Biochem.Biophys. Res.Comm. 90, 473-480.

Wood J.M., Sordahl L.A., Lewis R.M. and Schwartz A. (1973). Effect of chronic myocardial ischaemia on the activity of carnitine palmitoyl coenzyme A transferase of isolated canine heart mitochondria. Circ.Res. 32, 340-347.

Woodward B., Hassan M.N.M.Z., and Mohamed O.Y. (1983). Effects of potassium on ischaemic and reperfusion arrhythmias in the isolated rat heart. J.Mol.Cell.Cardiol. 15, supp I, 414.

Wurl M. and Kunze H. (1985). Purification and properties of phospholipase A_2 from human seminal plasma. Biochim.Biophys.Acta. 834, 411-418.

Yanagishita T., Katagiri T., Kitsu T., Geshi E., Konno N., Tanno F., Akiyama K., Sekita S., Kobayashi Y. and Niitani H. (1985). Degradation of phospholipids of sarcoplasmic reticulum in acute myocardial ischaemia. J.Mol.Cell.Cardiol. 17, supp I, 63.

Yanagishita T., Katagiri T., Mochizuki M., Umezu K., Kitsu E., Geshi E., Konno N., Tanno F., Akiyama K., Sekita S. and Niitani H. (1986). Changes in phospholipids in the acute ischaemic myocardium. J.Mol.Cell.Cardiol. 18, supp I, 133.

PUBLICATIONS

Bentham J.M., Higgins A.J. and Woodward B. (1985). Changes in lysophospholipids and phospholipase A_2 activity during ischaemia in the isolated rat heart. Br.J.Pharmac. 86, 810P.

Bentham J.M., Higgins A.J. and Woodward B. (1986). Effects of lipid amphiphiles on arrhythmogenesis in the anaesthetized cat. Br.J.Pharmac. 89, 735P.

Bentham J.M., Higgins A.J. and Woodward B. Phospholipase A_2 activity during ischaemia in the isolated rat heart. Basic.Res.Cardiol. (in press Proceedings supplement).

Presented at symposia

International Symposium on lipid metabolism in the normoxic and ischaemic heart 22/23 Sept. 1986.

Bentham J.M., Higgins A.J. and Woodward B. Phospholipase A_2 activity during ischaemia in the isolated rat heart.

Bentham J.M., Higgins A.J. and Woodward B. Involvement of lysophospholipids in arrhythmogenesis in the isolated rat heart and the anaesthetized cat.